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(54) Title: QUANTITATIVE ASSESSMENT OF erbB/HER RECEPTORS IN BIOLOGICAL FLUIDS

(57) Abstract: This invention provides a method for determining the amount of a protein in a biological sample, which protein has the following characteristics: (1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) induces nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein, which comprises: a) obtaining a biological sample from a subject; b) providing a cell comprising: (1) a first recombinant nucleic acid comprising a first DNA region encoding a transmembrane isoform of a neuregulin protein linked in frame to a second DNA region encoding a transcription factor wherein expression of the first recombinant nucleic acid in the cell produces a transmembrane isoform of a neuregulin protein-transcription factor fusion protein, and (2) a second recombinant nucleic acid comprising a promoter operatively linked to a reporter gene, wherein binding of the transcription factor portion of the fusion protein to the promoter activates expression of the reporter gene; c) contacting the biological sample with the cell; d) measuring reporter gene expression level in the cell; e) comparing the reporter gene expression level measured in step d) with a reporter gene expression level measured in multiple samples and multiple different known amounts of protein which selectively binds to a transmembrane isoform of a neuregulin protein, thereby determining the amount of a protein in a biological sample, which protein has the following characteristics: (1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) induces nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein. This invention further provides methods for detecting other functional molecules, methods for treating diseases, including neurodegenerative diseases and cancer, compounds and pharmaceutical compositions, and cells used in the methods described herein.

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Quantitative Assessment of erbB/HER Receptors  
In Biological Fluids

5 This invention is a continuation-in-part and claims the benefit of U.S. Serial No. 09/640,364, filed August 16, 2000, the contents of which are hereby incorporated by reference into this application.

10 The invention described herein was made with Government support under grant number NS29071 from the National Institutes of Health. Accordingly, the United States Government has certain rights in this invention.

Background of the Invention

15 Throughout this application, various publications are referenced by arabic numbers within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to  
20 more fully describe the state of the art to which this invention pertains. Full bibliographic citations for these references may be found listed numerically immediately preceding the claims.

25 Transmembrane neuregulin-1s (NRG-1) include an extracellular domain that is a ligand for erbB receptors and a highly conserved cytoplasmic domain of critical but unknown function. We demonstrate that the cytoplasmic domain of NRG-1 translocates to the nucleus and regulates  
30 gene expression in neurons. Nuclear translocation is induced either following interaction of the NRG-1 extracellular domain with erbB receptors or following

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membrane depolarization. Nuclear targeting requires the first eight intracellular amino acids immediately following the transmembrane domain. Thus transmembrane isoforms of NRG-1 act not only as growth factors but also  
5 as bi-directional signaling molecules.

Many isoforms of the neuregulin-1 gene (NRG-1) are membrane anchored growth factors consisting of an extracellular domain containing the ligand, a single  
10 transmembrane domain and a highly conserved cytoplasmic domain (1, 2). Interactions between the extracellular domain of NRG-1 and erbB receptor tyrosine kinases have been studied extensively (3, 4). In contrast the possible functions of the large and highly conserved  
15 cytoplasmic domains of NRG-1 are less clear.

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Summary of the Invention

This invention provides a method for determining the amount of a protein in a biological sample, which protein has the following characteristics: (1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) induces nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein, which comprises: a) obtaining a biological sample from a subject; b) providing a cell comprising: (1) a first recombinant nucleic acid comprising a first DNA region encoding a transmembrane isoform of a neuregulin protein linked in frame to a second DNA region encoding a transcription factor wherein expression of the first recombinant nucleic acid in the cell produces a transmembrane isoform of a neuregulin protein-transcription factor fusion protein, and (2) a second recombinant nucleic acid comprising a promoter operatively linked to a reporter gene, wherein binding of the transcription factor portion of the fusion protein to the promoter activates expression of the reporter gene; c) contacting the biological sample with the cell; d) measuring reporter gene expression level in the cell; e) comparing the reporter gene expression level measured in step d) with a reporter gene expression level measured in multiple samples and multiple different known amounts of protein which selectively binds to a transmembrane isoform of a neuregulin protein, thereby determining the amount of a protein in a biological sample, which protein has the following characteristics: (1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) induces nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein. This invention further provides methods for detecting



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other functional molecules, methods for treating diseases, including neurodegenerative diseases and cancer, compounds and pharmaceutical compositions, and cells used in the methods described herein.

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Brief Description of the Figures

Figure 1. Immunolocalization of NRG-1-CD in neurons.

(A) A low density E13.5 SGN culture was stained with an antibody recognizing an epitope in the c region of the NRG-1-CD (red), an antibody against MAP-2 (which stains both the neuronal cell body and the dendrites, shown in green) and with TOTO-3 (which stains the nucleus in blue). (B) Three color overlay images are shown of E13.5 SGNs (B1, one day in culture) and E16 hippocampal neurons (B2, three days in culture) stained with the antibody recognizing the a region of NRG-1-CD (red in B1, green in B2), neurofilament proteins (green in B1, red in B2) and TOTO-3 (blue). (C) High-density E13.5 SGNs cultures were treated with serbB2, a mixture of serbB2 and serbB4, or 50 mM KCl for 15 min. Cells were then fixed and stained with antibodies recognizing the a region of the NRG-1-CD (red), neurofilament proteins (NF, green) and TOTO-3 (NUCLEI, blue). In the merged images shown on the right, it is evident that combined treatment with serbB2 and serbB4 or depolarization with KCl resulted in an increase in punctate staining in nuclei. (D) The percent of nuclei showing punctate staining with the antibody recognizing the NRG-1-CD were quantified. In each case 2 independent images from each condition shown in panel C were analyzed for coincidence of red (NRG-1-CD) and blue (DNA) signals. Only cells showing clearly outlined nuclei were included in the analyses and at least 50 cells were scored per field. The data are plotted as the mean of the two counts. Treatment with serbB2 and serbB4 or with KCl resulted in a 4-5 fold increase in positive nuclei. (E) The neuronal processes stained by antibodies against neurofilaments (NF, green) and NRG-1-CD (red) were analyzed by a confocal microscopy. Less

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immunoreactivity of NRG-1-CD was observed after depolarization by KCl. (F) E13.5 SGNs from six cochleae were cultured overnight and then treated as described in panel C. Nuclear extracts were prepared and were  
5 analyzed by immunoblotting (18 mg extract protein/lane) with antibodies recognizing NRG-1-CDa, histone (a nuclear protein) or translational initiation factor 5 (TIF-5, a cytoplasmic protein). Consistent with the results shown in panel C and D, serbB2 + serbB4 or KCl treatments  
10 increased the amount of NRG-1-CDa in nuclei.

**Figure 2. Nuclear localization of NRG-1-CD in mammalian cells.** (A) Schematic diagram showing the domains of NRG-1Ba and NRG-1Bc forms, and the chimeric constructs used in  
15 this study. TM - transmembrane domain, JM - juxtamembrane region separating the TM from the site of proteolytic cleavage that releases soluble NRG, c and a - two alternate CD regions (note that a forms of NRG-1 have both the c region and the a region), NLS-1 and NLS-2 -  
20 the two putative nuclear localization sequences. Gal4-VP16 - chimeric transcription factor containing the Gal4 DNA binding domain fused to the VP16 activation domain, Myc and HA represent epitope tags, GFP - green fluorescent protein. (B & C) NRG-1Ba-Gal4-VP16 or Gal4-  
25 VP16 expressing plasmids were cotransfected into HEK293T cells with reporter plasmids containing 4 copies of the Gal4 UAS fused to either chloramphenicol acetyl transferase (pCAT, panel B) or luciferase (pLuc, panel C) coding regions. CAT and luciferase activities were  
30 measured 48 hr post-transfection (28, 29). Where indicated cells were treated with DMSO (0.1%) 4aPDD (0.5 mg/ml) or PMA (0.5 mg/ml) for the final 8 hrs prior to harvesting. (D) HEK293T cells transfected with NRG-1Ba-

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Myc were treated with DMSO or PMA for 15 min, fixed and stained with an antibody recognizing the Myc epitope (green) and with TOTO-3 (red). The punctate nuclear staining is indicated with the arrowhead. (E) HEK293T  
5 cells were transfected with NRG-1ba-GFP. Two days post-transfection live cells were observed with a fluorescence microscope. At T=0 min PMA and bisbenzimidazole were added and the cells were monitored for ~20 min. Images captured at 0 and 12 min are shown singly and following  
10 a computer overlay. (F) Particulate/membrane (P1 and P2), Nuclear (N1 and N2), and Cytoplasmic (C1 and C2) fractions were prepared from HEK293T cells transfected with the NRG-1ba-Myc expressing plasmid (P1, N1, and C1) or the control pCDNA3 plasmid (P2, N2, and C2) (30, 31).  
15 Proteins were analyzed by immunoblotting using antibodies recognizing the Myc epitope, histone or TIF5. Note two NRG bands above 79 kD labeled by "x", one NRG band near 50kD by "\*", and the endogenous myc band just below ~50 kD. (G) Nuclear fractions were prepared from HEK293T  
20 cells transfected with the NRG-1ba-HA expressing plasmid. Nuclear proteins were then analyzed by immunoblotting using an antibodies recognizing the HA epitope, histone or TIF5. Treatment of transfected cells for 15 min with PMA (0.5 mg/ml) resulted in a large increase in the  
25 amount of a protein of ~50 kD that was recognized by the HA antibody.

Figure 3. The NLS-1 functions as a nuclear targeting signal for the NRG-1-CD. HEK293T cells were transfected  
30 with plasmids expressing either NRG-1bc-CD-GFP or NRG-1bc-CD<sub>DNLS1</sub>-GFP (30). After 2 days cells were observed with a fluorescence microscope. Strong green fluorescence was seen in nuclei of cells expressing NRG-1bc-CD-GFP but not

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in nuclei of cells expressing NRG-1BC-CD<sub>DNL51</sub>-GFP.

Figure 4. Changes in gene expression following nuclear translocation of NRG-1-CD. (A) E13.5 SGNs from three cochleae were cultured overnight and then treated with serbB2 (lane 1), serbB2 and serbB4 (lane 2) or KCl (lane 3) for 2 hrs. Total RNA was isolated and the relative level of Bcl-X<sub>L</sub>, BAK, RIP, Oct-3, p19<sup>INK4</sup>, IL-11 and actin mRNAs were determined by RT-PCR. Amplified products were resolved on agarose gels and visualized with ethidium bromide. (B). The experiment described in panel A was repeated on total RNA from SGN cultures treated for 2 hrs with the extracellular domain of CRD-NRG-1B (20 mg/ml, lane 2), serbB2 (5 mg/ml) and serbB4 (5 mg/ml) pre-incubated with CRD-NRG-1B (10 mg/ml, lane 3) or serbB2 and serbB4 (20 mg/ml, lane 4), or left untreated (lane 1).

Figure 5. Disruption of CRD-NRG-1 Gene.  $\beta$ -form Nrg-1 ( $\beta$ -forms differ from  $\alpha$  forms in the EGF-like domain) splice variants and the various nomenclatures used in the literature are shown. "a", "b", and "c" refer to three alternative cytoplasmic (CYT) domains. "1", "2", and "3" designate three variants of the linker region connecting the EGF-like domain with the TM domain. Subclass 3 lacks a TM. Daggers indicate potential glycosylation sites. Abbreviation: EXT, extracellular.

Figure 6. Schematic showing the extracellular site of ligand-receptor interactions.

Figure 7. Schematic representing methods of detection and quantification provided by embodiments of this invention.

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Figure 8. Interaction with erbB2 and erbB4 or depolarization target NRG-1-ICD to the nucleus in primary neurons. (A) Dispersed E16 spiral ganglion neurons were maintained in vitro for 3 days and they were stained with antibodies recognizing the intracellular domain of the "a" form of NRG-1 (red), or neurofilaments (NF, green) and with TOTO-3 to label nuclei (blue). Fifteen minutes prior to fixation and staining, neuronal cultures were either untreated (control) or treated with soluble erbB2:B4 (serbB2:B4) or they were depolarized by adding 50 mM KCl to the medium. Inserts show selected neuronal soma/nuclei at higher power. (B) Enlarged color overlay images of neurons stained with the anti-NRG-1-ICD antibody (red) and TOTO-3 (blue). (C) The percent of neuronal nuclei showing staining with the antibody recognizing the NRG-1-ICD were quantified after 15 min treatment with nothing (control), soluble erbB2 (serbB2; which does not bind to NRG-1), soluble erbB2 and erbB4 (serbB2:B4) or 50 mM KCl. Only cells showing clearly outlined nuclei were included in the analyses and at least 50 cells were scored per field. The data are plotted as the mean of the counts from two experiments. (D) Spiral ganglion neurons from 6 E13.5 embryos were maintained in culture overnight prior to treatment for 15 min with nothing, serbB2, serbB2:B4 or 50 mM KCl. Cytoplasmic and nuclear extracts (18  $\mu$ g protein/lane) were resolved by SDS-PAGE and NRG-1-ICD was detected by probing immunoblots with the ICD antibody. After stripping the filters were reprobed sequentially with antibodies recognizing histone H1(H1) or the translation initiation factor eIF5). The anti-NRG-1-ICD antibody recognized proteins of  $M_w$  102 kD and 50 kD. (E) Nuclei from cells treated with serbB2:B4 or 50 mM KCl had

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significantly elevated levels of the 50 kD band.

**Figure 9.** Localization and functional analysis of NRG-1-ICD in transfected cells. (A) Schematic diagram showing NRG-1 $\beta$ a and the chimeric constructs used in this study. TM - transmembrane domain, JM - Juxtamembrane region involved in metalloprotease cleavage and release of the extracellular EGF-like peptide. NLS— the putative nuclear localization sequence. GFP— green fluorescent protein. HA - influenza virus hemagglutinin derived epitope tag. Gal4-VP16— chimeric transcription factor containing the yeast Gal4 DNA binding domain fused to the herpesvirus protein VP16 activation domain. Gal4<sub>DBD</sub>— DNA binding domain of Gal4. (B) Intracellular movement of NRG-1 $\beta$ a-GFP was followed in live cells by two-photon microscopy. Images (1  $\mu$ m thick sections through the center of the nucleus) were collected at various intervals (in minutes) following treatment with soluble erbB2 and erbB4 (erbB2:B4). (C) Cytoplasmic (Cyto.) and Nuclear (Nuc.) fractions were prepared from mock transfected or NRG-1 $\beta$ a-HA transfected HEK293T cells (30,31). Proteins were analyzed by immunoblotting using antibodies recognizing the HA epitope. NRG-1 $\beta$ a-HA transfected cells were treated for 15 min with soluble erbB2 (32  $\mu$ g/ml) or soluble erbB2:4 (32  $\mu$ g/ml). In addition to a doublet of non-specific (NS) bands, proteins of greater than 100 kD (full length and aggregated NRG-1 $\beta$ a-Gal4-VP16 or ID-Gal4<sub>DBD</sub> expression plasmids were cotransfected into HEK293T cells with a reporter plasmid containing 4 copies of the Gal4 UAS fused to the luciferase coding region. Luciferase activities were measured 48 hr post-transfection (28,29).

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**Figure 10.** NRG-1 processing requires  $\gamma$ -secretase activity. HEK293T cells were transfected with NRG-1 $\beta$ a-GFP. Two days post-transfection cells were treated with an inhibitor of  $\gamma$ -secretase ( $\gamma$ -Sec I) and observed with a two-photon microscope. Bisbenzimidazole was added to label nuclei. At T=0 min, images of transfected cells at the middle level of their nuclei were recorded, and where indicated serbB2:B4 (40  $\mu$ g/ml) was added to the culture medium. The cells were continually monitored for additional 40 min with no apparent changes in NRG-1 $\beta$ a-GFP localization. Images from the T=0 and T=20 min time points are shown. (B) Whole cell lysates (30  $\mu$ g protein/lane) from NRG-1 $\beta$ a-HA transfected HEK293T were analyzed by immunoblotting using antibodies recognizing the HA epitope. Transfected cells were untreated (lanes 1 and 2) or pretreated with a  $\gamma$ -secretase for 8 hr ( $\gamma$ -Sec I, lanes 3 and 4) prior to stimulation with serbB2 alone (lanes 1 and 3) or serbB2:B4 (lanes 2 and 4) for 15 min. Pretreatment with the  $\gamma$ -secretase inhibitor resulted in a massive increase of predominantly full length NRG-1 $\beta$ a-HA (ECL exposure was for 5 s). (C) An NRG-1 $\beta$ a-Gal4-VP16 expressing plasmid was cotransfected into HEK293T cells with the Gal4-UAS-luciferase reporter. Luciferase activity was measured 48 hr post-transfection (28, 29). Twenty-four hr post-transfection cells were treated with a  $\gamma$ -secretase inhibitor ( $\gamma$ -Sec I) or an inactive analogue of the inhibitor (mock  $\gamma$ -Sec I). Eight hr prior to measuring luciferase activity cells were treated with soluble erbB2 (serbB2), soluble erbB2 and erbB4 (serbB2:B4), serbB2:B4 pre-incubated with the extracellular domain of CRD-NRG-1 (ECD), or the CRD-NRG-1 ECD (ECD) alone.



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**Figure 11.** Stimulated nuclear targeting of NRG-1-ICD alters neuronal gene expression and protects from apoptosis. (A) E13.5 spiral ganglion neurons maintained in culture overnight were untreated (control, lane 2) or stimulated with serbB2:B4 (lane 3) or 50 mM KCl (lane 4) for 2 hrs. Total RNA was isolated and the relative level of Bcl-X<sub>L</sub>, BAK, RIP, Oct-3, p19<sup>INK4</sup>, and actin mRNAs were determined by RT-PCR. Amplified products were resolved on agarose gels and visualized with ethidium bromide. In (B) this experiment was repeated except that additional neurons were stimulated with serbB2:B4 that had been pre-incubated with the extracellular domain of CRD-NRG-1 (lane 4). (C and D) Dispersed neurons were maintained in culture for two days, untreated or pretreated with  $\gamma$ -secretase inhibitor ( $\gamma$ -Sec I; 0.2  $\mu$ M MW111-26A) for 8 hours, and then stimulated with serbB2:B4, CRD-NRG-ECD, or a mixture of serbB2:4 and NRG-ECD (40  $\mu$ g/ $\mu$ l) for an additional 8 hours. Apoptotic cells were visualized (c) by staining with nuclei with bisbenzimidazole. The percentage of total nuclei that appeared apoptotic was quantified in three independent experiments (D). Ten samples were taken for one group from each independent study. Where indicated (\*) values differed significantly from the untreated control group ( $p < 0.01$ ).

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**Figure 12.** Bi-directional signaling by transmembrane NRG-1. Both forward and back signaling result from interactions between erbB receptors and membrane tethered NRG-1. Interaction results in activation of erbB receptor tyrosine kinases and subsequent induction of target genes, including nicotinic acetylcholine receptor (AChR) subunits. In addition,  $\gamma$ -secretase dependent processing of NRG-1 releases the ICD which translocates

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to the nucleus and regulates target gene expression.

**Detailed Description of the Invention**

5 This invention provides a cell comprising (1) a first recombinant nucleic acid comprising a first DNA region encoding a transmembrane isoform of a neuregulin protein linked in frame to a second DNA region encoding a transcription factor wherein expression of the first recombinant nucleic acid in the cell produces a  
10 transmembrane isoform of a neuregulin protein-transcription factor fusion protein, and (2) a second recombinant nucleic acid comprising a promoter operatively linked to a reporter gene, wherein binding of the transcription factor portion of the fusion protein to  
15 the promoter activates expression of the reporter gene.

This invention provides a method for detecting the presence of a protein in a biological sample, which protein has the following characteristics: (1)  
20 selectively binds to a transmembrane isoform of a neuregulin protein and (2) induces nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein, which comprises: a) obtaining a biological sample from a subject; b) providing a cell  
25 comprising: (1) a first recombinant nucleic acid comprising a first DNA region encoding a transmembrane isoform of a neuregulin protein linked in frame to a second DNA region encoding a transcription factor wherein expression of the first recombinant nucleic acid in the  
30 cell produces a transmembrane isoform of a neuregulin protein-transcription factor fusion protein, and (2) a second recombinant nucleic acid comprising a promoter operatively linked to a reporter gene, wherein binding of

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the transcription factor portion of the fusion protein to the promoter activates expression of the reporter gene; c) contacting the biological sample with the cell; d) measuring reporter gene expression level in the cell, wherein an increased reporter gene expression level compared to the reporter gene expression level measured in the cell in the absence of the biological sample is indicative of the presence of a protein in the biological sample, which protein has the following characteristics:

(1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) induces nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein.

This invention provides a method for determining the amount of a protein in a biological sample, which protein has the following characteristics: (1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) induces nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein, which comprises: a) obtaining a biological sample from a subject; b) providing a cell comprising: (1) a first recombinant nucleic acid comprising a first DNA region encoding a transmembrane isoform of a neuregulin protein linked in frame to a second DNA region encoding a transcription factor wherein expression of the first recombinant nucleic acid in the cell produces a transmembrane isoform of a neuregulin protein-transcription factor fusion protein, and (2) a second recombinant nucleic acid comprising a promoter operatively linked to a reporter gene, wherein binding of the transcription factor portion of the fusion protein to the promoter activates expression of the reporter gene;

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c) contacting the biological sample with the cell; d) measuring reporter gene expression level in the cell; e) comparing the reporter gene expression level measured in step d) with a reporter gene expression level measured in multiple samples and multiple different known amounts of protein which selectively binds to a transmembrane isoform of a neuregulin protein, thereby determining the amount of a protein in a biological sample, which protein has the following characteristics: (1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) induces nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein.

This invention provides a method for early detection of cancer in a subject which comprises: a) obtaining a biological sample from a first subject; b) providing a cell comprising: 1) a first recombinant nucleic acid comprising a first DNA region encoding a transmembrane isoform of a neuregulin protein linked in frame to a second DNA region encoding a transcription factor wherein expression of the first recombinant nucleic acid in the cell produces a transmembrane isoform of a neuregulin protein-transcription factor fusion protein, and (2) a second recombinant nucleic acid comprising a promoter operatively linked to a reporter gene, wherein binding of the transcription factor portion of the fusion protein to the promoter activates expression of the reporter gene; c) contacting the biological sample with the cell; d) measuring reporter gene expression level in the cell; e) comparing the reporter gene expression level in d) with a reporter gene expression level measured in a biological sample which is from a second subject without cancer, wherein a higher reporter gene expression level

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in the biological sample from the first subject is indicative of the first subject having cancer.

This invention provides a method for identifying a  
5 compound, which compound has the following characteristics: (1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) induces nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein, which  
10 comprises: a) admixing a compound with a cell comprising: (1) a first recombinant nucleic acid comprising a first DNA region encoding a transmembrane isoform of a neuregulin protein linked in frame to a second DNA region encoding a transcription factor wherein expression of the  
15 first recombinant nucleic acid in the cell produces a transmembrane isoform of a neuregulin protein-transcription factor fusion protein, and (2) a second recombinant nucleic acid comprising a promoter operatively linked to a reporter gene, wherein binding of  
20 the transcription factor portion of the fusion protein to the promoter activates expression of the reporter gene; b) measuring reporter gene expression level in the cell, wherein an increased reporter gene expression level compared to the reporter gene expression level in the  
25 cell in the absence of the compound is indicative of a compound having the following characteristics: (1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) induces nuclear translocation of a cytoplasmic domain of the transmembrane isoform of  
30 the neuregulin protein.

This invention provides a method for identifying a compound, which compound has the following

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characteristics: (1) selectively binds to a transmembrane isoform of a neuregulin protein in a cell and (2) inhibits nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein in a cell, which comprises: a) admixing a compound with a cell transfected with (1) a recombinant nucleic acid comprising a first DNA sequence encoding a transmembrane isoform of a neuregulin protein linked in frame to a second DNA sequence encoding a transcription factor wherein expression of the recombinant nucleic acid produces a fusion protein, and (2) a recombinant nucleic acid comprising a promoter region operatively linked to a reporter gene, wherein the transcription factor portion of the fusion protein binds to the promoter region thereby activating expression of the reporter gene; b) measuring reporter gene expression level in the cell, wherein a decreased reporter gene expression level compared to the reporter gene expression level in the cell in the absence of the compound is indicative of the presence of a compound, which compound has the following characteristics: (1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) inhibits nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein.

This invention provides a cell which comprises (1) a first recombinant nucleic acid comprising a first DNA region encoding a ligand binding domain of a protein linked in frame to a second DNA sequence encoding a channel forming domain a  $\alpha$ -7 type neuronal nicotine receptor linked in frame to a third DNA region encoding a transcription factor wherein expression of the first recombinant nucleic acid produces a ligand binding domain

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of a protein-channel forming domain a  $\alpha$ -7 type neuronal  
nicotine receptor-transcription factor fusion protein and  
(2) a second recombinant nucleic acid comprising a  
promoter operatively linked to a reporter gene, wherein  
5 the transcription factor binds to the promoter thereby  
activating expression of the reporter gene.

This invention provides a method for detecting the  
presence of a molecule in a biological sample, which  
10 molecule selectively binds to a ligand gated ion channel  
receptor, which comprises: a) obtaining a biological  
sample from a subject; b) contacting the biological  
sample with a cell which comprises (1) a first  
recombinant nucleic acid comprising a first DNA region  
15 encoding a ligand binding domain of a protein linked in  
frame to a second DNA sequence encoding a channel forming  
domain a  $\alpha$ -7 type neuronal nicotine receptor linked in  
frame to a third DNA region encoding a transcription  
factor wherein expression of the first recombinant  
20 nucleic acid produces a ligand binding domain of a  
protein-channel forming domain a  $\alpha$ -7 type neuronal  
nicotine receptor-transcription factor fusion protein and  
(2) a second recombinant nucleic acid comprising a  
promoter operatively linked to a reporter gene, wherein  
25 the transcription factor binds to the promoter thereby  
activating expression of the reporter gene; c) measuring  
reporter gene expression level in the cell, wherein an  
increased reporter gene expression level compared to the  
reporter gene expression level measured in the cell in  
30 the absence of the biological sample is indicative of  
the presence of a molecule which selectively binds to a  
ligand gated ion channel receptor in a biological sample.

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This invention provides a method for determining the amount of a molecule in a biological sample, which molecule selectively binds to a ligand gated ion channel receptor, which comprises: a) obtaining a biological sample from a subject; b) providing a cell which comprises (1) a first recombinant nucleic acid comprising a first DNA region encoding a ligand binding domain of a protein linked in frame to a second DNA sequence encoding a channel forming domain a  $\alpha$ -7 type neuronal nicotine receptor linked in frame to a third DNA region encoding a transcription factor wherein expression of the first recombinant nucleic acid produces a ligand binding domain of a protein-channel forming domain a  $\alpha$ -7 type neuronal nicotine receptor-transcription factor fusion protein and (2) a second recombinant nucleic acid comprising a promoter operatively linked to a reporter gene, wherein the transcription factor binds to the promoter thereby activating expression of the reporter gene; c) contacting the biological sample with the cell; d) measuring reporter gene expression level; e) comparing the reporter gene expression level measured in step d) with a reporter gene expression level measured in multiple samples and multiple different known amounts of molecule which selectively binds to a ligand gated ion channel receptor, thereby determining the amount of a molecule, which molecule selectively binds to a ligand gated ion channel receptor.

This invention provides a method for early detection of a neurodegenerative disease in a subject which comprises: a) obtaining a biological sample from a first subject; b) contacting the sample with a cell which comprises (1) a first recombinant nucleic acid comprising



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a first DNA region encoding a ligand binding domain of a protein linked in frame to a second DNA sequence encoding a channel forming domain a  $\alpha$ -7 type neuronal nicotine receptor linked in frame to a third DNA region encoding a transcription factor wherein expression of the first recombinant nucleic acid produces a ligand binding domain of a protein-channel forming domain a  $\alpha$ -7 type neuronal nicotine receptor-transcription factor fusion protein and

5 a transcription factor wherein expression of the first recombinant nucleic acid produces a ligand binding domain of a protein-channel forming domain a  $\alpha$ -7 type neuronal nicotine receptor-transcription factor fusion protein and

(2) a second recombinant nucleic acid comprising a promoter operatively linked to a reporter gene, wherein the transcription factor binds to the promoter thereby activating expression of the reporter gene; c) measuring reporter gene expression level in the cell; d) comparing the reporter gene expression level in c) with a reporter gene expression level in a sample which is from a second subject without neurodegenerative disease, a lower amount in the sample from the first subject being indicative of the first subject having a neurodegenerative disease.

10 the transcription factor binds to the promoter thereby activating expression of the reporter gene; c) measuring reporter gene expression level in the cell; d) comparing the reporter gene expression level in c) with a reporter gene expression level in a sample which is from a second subject without neurodegenerative disease, a lower amount in the sample from the first subject being indicative of the first subject having a neurodegenerative disease.

15 gene expression level in a sample which is from a second subject without neurodegenerative disease, a lower amount in the sample from the first subject being indicative of the first subject having a neurodegenerative disease.

20 This invention provides a cell transfected with (1) a recombinant nucleic acid comprising a first DNA sequence encoding a transmembrane isoform of a neuregulin protein linked in frame to a second DNA sequence encoding a transcription factor wherein expression of the recombinant nucleic acid produces a fusion protein, and

25 (2) a recombinant nucleic acid comprising a promoter region operatively linked to a reporter gene, wherein the transcription factor portion of the fusion protein binds to the promoter region thereby activating expression of the reporter gene.

30 the reporter gene.

This invention provides a method for detecting the presence of a protein in a biological sample, which

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protein has the following characteristics: (1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) induces nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein, which comprises: a) obtaining a biological sample from a subject; b) providing a cell transfected with (1) a recombinant nucleic acid comprising a first DNA sequence encoding a transmembrane isoform of a neuregulin protein linked in frame to a second DNA sequence encoding a transcription factor wherein expression of the recombinant nucleic acid produces a fusion protein, and (2) a recombinant nucleic acid comprising a promoter region operatively linked to a reporter gene, wherein the transcription factor portion of the fusion protein binds to the promoter region thereby activating expression of the reporter gene; c) contacting the biological sample with the cell; d) measuring reporter gene expression level in the cell, wherein an increased reporter gene expression level compared to the reporter gene expression level in the cell in the absence of the biological sample is indicative of the presence of a protein in the biological sample, which protein has the following characteristics: (1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) induces nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein.

This invention provides a method for determining the amount of a protein in a biological sample, which protein has the following characteristics: (1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) induces nuclear translocation of a cytoplasmic domain

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of the transmembrane isoform of the neuregulin protein, which comprises: a) obtaining a biological sample from a subject; b) providing a cell transfected with (1) a recombinant nucleic acid comprising a first DNA sequence  
5 encoding a transmembrane isoform of a neuregulin protein linked in frame to a second DNA sequence encoding a transcription factor wherein expression of the recombinant nucleic acid produces a fusion protein, and (2) a recombinant nucleic acid comprising a promoter  
10 region operatively linked to a reporter gene, wherein the transcription factor portion of the fusion protein binds to the promoter region thereby activating expression of the reporter gene; c) contacting the biological sample with the cell; d) measuring reporter gene expression  
15 level in the cell; e) comparing the reporter gene expression level in d) with a reporter gene expression level measured in a sample with a known amount of protein which selectively binds to a transmembrane isoform of a neuregulin protein, thereby determining the amount of a  
20 protein in a biological sample, which protein has the following characteristics: (1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) induces nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein.

25 This invention provides a method for early detection of cancer in a subject which comprises: a) obtaining a biological sample from a first subject; b) providing a cell transfected with (1) a recombinant nucleic acid  
30 comprising a first DNA sequence encoding a transmembrane isoform of a neuregulin protein linked in frame to a second DNA sequence encoding a transcription factor wherein expression of the recombinant nucleic acid

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produces a fusion protein, and (2) a recombinant nucleic acid comprising a promoter region operatively linked to a reporter gene, wherein the transcription factor portion of the fusion protein binds to the promoter region thereby activating expression of the reporter gene;

5 c) contacting the biological sample with the cell;

d) measuring reporter gene expression level in the cell;

e) comparing the reporter gene expression level in d) with a reporter gene expression level measured in a biological sample which is from a second subject without

10 cancer, a higher reporter gene expression level in the biological sample from the first subject being indicative of the first subject having cancer.

15 This invention provides a method for identifying a compound, which compound has the following characteristics: (1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) induces nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein, which

20 comprises: a) admixing a compound with a cell transfected with (1) a recombinant nucleic acid comprising a first DNA sequence encoding a transmembrane isoform of a neuregulin protein linked in frame to a second DNA

25 sequence encoding a transcription factor wherein expression of the recombinant nucleic acid produces a fusion protein, and (2) a recombinant nucleic acid comprising a promoter region operatively linked to a reporter gene, wherein the transcription factor portion

30 of the fusion protein binds to the promoter region thereby activating expression of the reporter gene; b) measuring reporter gene expression level in the cell, wherein an increased reporter gene expression level

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compared to the reporter gene expression level in the cell in the absence of the compound is indicative of the presence of a compound, which compound has the following characteristics: (1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) induces nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein.

This invention provides a method for identifying a compound, which compound has the following characteristics: (1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) inhibits nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein, which comprises: a) admixing a compound with a cell transfected with (1) a recombinant nucleic acid comprising a first DNA sequence encoding a transmembrane isoform of a neuregulin protein linked in frame to a second DNA sequence encoding a transcription factor wherein expression of the recombinant nucleic acid produces a fusion protein, and (2) a recombinant nucleic acid comprising a promoter region operatively linked to a reporter gene, wherein the transcription factor portion of the fusion protein binds to the promoter region thereby activating expression of the reporter gene; b) measuring reporter gene expression level in the cell, wherein a decreased reporter gene expression level compared to the reporter gene expression level in the cell in the absence of the compound is indicative of the presence of a compound, which compound has the following characteristics: (1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) inhibits nuclear translocation of a cytoplasmic domain of the

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transmembrane isoform of the neuregulin protein.

This invention provides a pharmaceutical composition comprising: i) a compound which (1) selectively binds to  
5 a transmembrane isoform of a neuregulin protein and (2) inhibits nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein, determined to do so by the methods described herein; and ii) a pharmaceutically acceptable carrier.

10

This invention provides a method for treating cancer in a subject which comprises administering to the subject a therapeutically effective amount of a compound which compound has the following characteristics: (1)  
15 selectively binds to a transmembrane isoform of a neuregulin protein and (2) inhibits nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein or a pharmaceutical composition of comprising: i) a compound which (1) selectively binds to  
20 a transmembrane isoform of a neuregulin protein and (2) inhibits nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein, determined to do so by the methods described herein; and ii) a pharmaceutically acceptable carrier so as to treat  
25 cancer in a subject.

In an embodiment of the invention, the cell described herein is a cell wherein the reporter gene encodes a green fluorescent protein, a  $\beta$ -galactosidase, a  
30 luciferase, a chloramphenicol acetyltransferase, a  $\beta$  glucuronidase, a neomycin phosphotransferase, or a guanine xanthine phosphoribosyltransferase.

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In an embodiment of the invention, the methods described herein are methods wherein the reporter gene encodes a green fluorescent protein, a  $\beta$ -galactosidase, a luciferase, a chloramphenicol acetyltransferase, a  $\beta$  glucuronidase, a neomycin phosphotransferase, or a guanine xanthine phosphoribosyltransferase.

In an embodiment of the invention, the protein of the methods above is an ErbB1/HER1 ErbB2/HER2, ErbB3/HER3, or ErbB4/HER4.

In an embodiment of the invention, the transmembrane isoform of the neuregulin is a cysteine rich domain neuregulin (CRD-NRG).

In an embodiment of the invention, the cytoplasmic domain of the transmembrane isoform of the neuregulin is a cyt-a, cyt-b, or cyt-c domain.

In an embodiment of the invention, the promoter is a gal4 upstream activator sequence or other promoter known in the art.

In an embodiment of the invention, the transcription factor is a gal4/VP16 transcription factor or other transcription factor known in the art.

In an embodiment of the invention, the cell is a human embryonic kidney cell.

In an embodiment of the invention, the cancer is a breast cancer, an ovarian cancer, a prostate cancer, a glioma, or a neuroblastoma.

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This invention provides a cell transfected with (1) a recombinant nucleic acid comprising a first DNA sequence encoding a ligand binding domain linked in frame to a second DNA sequence encoding a channel forming domain  
5 linked to a third DNA sequence encoding a transcription factor wherein expression of the recombinant nucleic acid produces a fusion protein and a transcription factor and (2) a recombinant nucleic acid comprising a promoter region operatively linked to a reporter gene, wherein the  
10 transcription factor binds to the promoter region thereby activating expression of the reporter gene.

This invention provides a method for detecting the presence of a molecule in a biological sample, which  
15 molecule selectively binds to a ligand gated ion channel receptor, which comprises: a) obtaining a biological sample from a subject; b) contacting the biological sample with a cell transfected with (1) a recombinant nucleic acid comprising a first DNA sequence encoding a  
20 ligand binding domain linked in frame to a second DNA sequence encoding a channel forming domain linked to a third DNA sequence encoding a transcription factor wherein expression of the recombinant nucleic acid produces a fusion protein and a transcription factor and  
25 (2) a recombinant nucleic acid comprising a promoter region operatively linked to a reporter gene, wherein the transcription factor binds to the promoter region thereby activating expression of the reporter gene; c) measuring reporter gene expression level in the cell, wherein an  
30 increased reporter gene expression level compared to the reporter gene expression level in the cell in the absence of the biological sample is indicative of the presence of a molecule which selectively binds to a ligand gated



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ion channel receptor in a biological sample.

This invention provides a method for determining the amount of a molecule in a biological sample, which molecule selectively binds to a ligand gated ion channel receptor, which comprises: a) obtaining a biological sample from a subject; b) providing a cell transfected with (1) a recombinant nucleic acid comprising a first DNA sequence encoding a ligand binding domain linked in frame to a second DNA sequence encoding a channel forming domain linked to a third DNA sequence encoding a transcription factor wherein expression of the recombinant nucleic acid produces a fusion protein and a transcription factor and (2) a recombinant nucleic acid comprising a promoter region operatively linked to a reporter gene, wherein the transcription factor binds to the promoter region thereby activating expression of the reporter gene; c) contacting the biological sample with the cell; d) measuring reporter gene expression level; e) comparing the reporter gene expression level in d) with a reporter gene expression level measured in a sample with a known amount of molecule which selectively binds to a ligand gated ion channel receptor, thereby determining the amount of a molecule, which molecule selectively binds to a ligand gated ion channel receptor.

This invention provides a method for early detection of a neurodegenerative disease in a subject which comprises: a) obtaining a biological sample from a first subject; b) contacting the sample with a cell transfected with (1) a recombinant nucleic acid comprising a first DNA sequence encoding a ligand binding domain linked in frame to a second DNA sequence encoding a channel forming domain

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linked to a third DNA sequence encoding a transcription factor wherein expression of the recombinant nucleic acid produces a fusion protein and a transcription factor and

(2) a recombinant nucleic acid comprising a promoter  
5 region operatively linked to a reporter gene, wherein the transcription factor binds to the promoter region thereby activating expression of the reporter gene; c) measuring reporter gene expression level in the cell; d) comparing the reporter gene expression level in c) with a reporter  
10 gene expression level in a sample which is from a second subject without neurodegenerative disease, a lower amount in the sample from the first subject being indicative of the first subject having a neurodegenerative disease.

15 In an embodiment of the invention, the ligand binding domain specifically binds to a neuregulin receptor, neurotransmitter, or neurotransmitter metabolite.

In an embodiment of the invention, the channel forming  
20 domain is a calcium channel forming domain of a  $\alpha$ -7 type neuronal nicotine receptor.

In an embodiment of the present invention the biological sample is blood, cerebrospinal fluid (CSF), plasma,  
25 sputum, amniotic fluid, ascites fluid, breast aspirate, saliva, urine, lung lavage, or cell lysate or extract derived from a biopsy.

In an embodiment of the invention, the cell is a human  
30 embryonic kidney cell.

In an embodiment of the invention, the promoter region is a CRE binding site, or other promoter known in the art.

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In an embodiment of the invention, the transcription factor is a CREB transcription factor, or other transcription factor known in the art.

5 In an embodiment of the invention, the neurodegenerative disease is Alzheimer's disease or Parkinson's Disease. In an embodiment of the invention, the neurodegenerative disease is associated with aging, amyotrophic lateral sclerosis, dentatorubral and pallidolysian atrophy,  
10 Huntington's disease, Machado-Joseph disease, multiple sclerosis, muscular dystrophy, senility, spinocerebellar ataxia type I, spinobulbar muscular atrophy, stroke, trauma.

15

In an embodiment of the present invention, the cell is a bacterial cell, a yeast cell, a fungal cell, an insect cell, a nematode cell, a plant or animal cell.

20 In an embodiment of the invention, the carrier of the pharmaceutical composition described herein comprises saline, sodium acetate, ammonium acetate, a virus, a liposome, a microencapsule, a polymer encapsulated cell, a retroviral vector, a diluent, or an isotonic,  
25 pharmaceutically acceptable buffer solution.

In an embodiment of the invention, the compound of the methods described herein is a peptide, a peptidomimetic, a nucleic acid, an organic molecule, an inorganic  
30 chemical, or a lipid-based compound.

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This invention provides a method for treating a neurodegenerative disease in a subject which comprises administering to the subject a therapeutically effective amount of a compound described herein, which compound has the following characteristics: (1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) induces nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein described herein, and a carrier, so as to treat a neurodegenerative disease in a subject.

In an embodiment of the invention, the subject is a mammal. In an embodiment, the mammal is a human.

In an embodiment of the invention, the molecule of the cell and methods described hereinabove is a neuregulin receptor, a neurotransmitter, or a neurotransmitter metabolite.

In an embodiment, the administering of the pharmaceutical compositions and compounds to a subject is via intralesional, intraperitoneal, intramuscular or intravenous injection; infusion; liposome-mediated delivery; topical, nasal, oral, anal, ocular or otic delivery.

Signaling between neuregulins and erbB receptors plays an important role in the normal development of many tissues. Dysregulation of this signaling is a feature of several human cancers, including cancer of the breast, ovary and prostate. For example, more than 30% of breast cancers

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express highly-elevated levels of erbB2, and 20-30%  
express elevated erbB3. Overexpression in tumor cells is  
associated with a poorer prognosis but also indicates  
that a patient might respond to the recently-approved  
5 Herceptin therapy. Recently, we have discovered a novel  
aspect of neuregulin-erbB interactions. Interaction  
between transmembrane forms of neuregulin and erbB  
receptors, both expressed on target cell membranes, and  
soluble, results in cleavage of the neuregulin with  
10 subsequent translocation of the cytoplasmic domain to the  
nucleus.

This invention provides a method for detection and  
quantification of erbBs in biological fluids. The  
15 following indicator cell line is used: a human embryonic  
kidney cell (293T) expressing a fusion protein between  
neuregulin and a chimeric Gal4/VP16 transcription factor  
and containing a reporter gene in which green fluorescent  
protein expression is regulated by tandem copies of the  
20 Gal4 upstream activator sequence. Reporter cells grown  
over night in low serum media are exposed to test  
solutions for 12-18 hrs. Cultures are then analyzed by  
fluorescence activated cell sorting. Both the total  
number of fluorescent cells and the fluorescence  
25 intensity are measured. The level of GFP expression is  
proportional to the level of the NRG-Gal4/VP16 fusion  
protein in the nucleus. This in turn is proportional to  
the level of erbB receptors in the culture media. Both  
values are compared against those obtained with known  
30 amounts of soluble erbB2 and erbB4.

This invention is useful as a diagnostic assay for

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soluble neuregulin receptors present in blood associated with erbB overexpression in human tumors. It is also useful in the development of therapeutics and compound and compositions for treating diseases including cancer and neurodegenerative diseases.

A problem which this invention solves is that it provides a highly-sensitive, quantitative assay of functional erbB/HER receptors in the bloodstream of cancer patients.

This invention provides an improvement over ELISA assays and differs from the closest prior art in that it distinguishes between functional and non-functional protein, including, erbB/HER protein.

This invention provides the following advantages, it assays the functional protein level in biological fluids rather than antigenic fragments; it has high specificity because ligand-receptor interactions are required for readout; it quantifies the types of functional and non-functional erbB/HER receptors present in the test fluids (i.e. distinguishes between erbB2, HER2, erbB3/HER 3, and erbB4/HER4); wherein interaction of erbBs with neuregulins leading to nuclear translocation of the neuregulin cytoplasmic domain has relevance to the etiology of specific cancers (e.g. breast, ovarian, and prostate), the invention has potential for development of novel pharmacological or genetic therapeutics.

This invention provides signaling based assays to assay clinically relevant (i.e. active) pools of neurotrophic factors, their receptors or neurotransmitters.

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Basic Neuregulin Structure/function

Some types are synthesized and directly secreted by cells. Some subtypes are synthesized as transmembrane proteins that have distinct extracellular, transmembrane  
5 and intracellular domains.

Alpha neuregulins predominate in "real" tissues, beta neuregulins predominate in nervous system.

10 Basic erb/her structure/function

Four neuregulin receptor subunits have been identified to date: called erb 1,2,3,4 or her 1,2,3 and 4. Erbs are single pass, transmembrane proteins; extracellular domain binds ligands, intracellular domains initiate signal  
15 transduction. Erbs are ligand dependent tyrosine kinases. Erb ligands induce dimerization.

Neuregulin-erb interactions

Nrg bind to erb/her. Binding activates forward  
20 signaling. This involves activation of the erb/her tyrosine kinase activity. Binding also activates back signaling. This involves nuclear translocation of the nrg cytoplasmic domain. Therefore, nrg acts as both ligand and receptor.

25

This invention exploits recent discoveries re: the role of both forward and back signalling in nrg-erb interactions. Targeting of the nrg in normal cells & tumors is monitored with a nrg-gal4/vp16 chimeric  
30 transcription factor and a gal4-gfp reporter gene. Reporter gene expression will only occur when the

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chimeric transcription factor is in the nucleus. Nuclear translocation of the chimeric transcription factor is specifically and selectively evoked by soluble erb/hers. The amount and types of functional erbs in the test sample is monitored by facs analysis of the number of gfp + detector cells and the profile of floresence intensity. The reporter gene may be other than gfp.

In addition to providing a rapid, sensitive and readily quantifiable measure of soluble erb/hers in biological fluids, the invention provides for a means of testing for potential therapeutic agents that inhibit or activate erb-nrg signaling.

The invention also utilizes the specificity of neurotransmitter and neurotrophic factor binding by well characterized receptor proteins and the ability to make and express functional, chimeric constructs of ligand binding domains linked to the channel forming domains of the  $\alpha 7$ -type neuronal nicotine receptor. The invention also exploits the fact that wt and chimeric  $\alpha 7$ -type subunits form functional channels that are highly permeable to calcium.

In an embodiment of the invention, ligand specific binding and activation of the chimeric  $\alpha 7$  receptors is monitored by ca-dependent creb and a cre-gfp reporter construct.

In one embodiment of the present invention, gfp expression will only occur when the chimeric cre-gfp



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reporter construct is activated by ligand-gated calcium influx.

5 In one embodiment, the amount and types of functional erbs, neuregulin(s), neurotransmitter or neurotransmitter metabolite in the test sample is monitored by facs analysis. (i.e. the number of gfp + detector cells and the profile of gfp fluorescence intensity)

10 Nrg/erb interactions in disease

Cancer:

Erb/hers are over-expressed and highly active in cancers of the breast, ovary and prostate.

15 Erb/her involvement in other cancers including gliomas & neuroblastomas is also likely.

Evidence:

20 Erb/her overexpression is associated with the presence of soluble erb/hers in the circulation.

Preliminary studies suggest that antibodies to human erb/her2 kill human breast cancer cells in which erb/her2 is overexpressed.

25

The pattern of expression of nrgs is abnormal in breast and prostate carcinoma: transformed cells can express nrg and nrg receptors.

30

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Neurodegenerative diseases:

CRD NRG is expressed in specific subsets of CNS and PNS neurons. CRD-NRG mRNA is detected immediately following emigration of newly born neurons from the ventricular zone. CRD-NRG protein is detected in axons of developing neurons. The expression of CRD NRG is required for the maintenance of newly formed synapses.

In CRD-NRG (-/-) mice, neurons that normally express this isoform extend to their target fields & initiate early pre and post synaptic specialization. However, these early interactions are not sustained. Following an overtly aberrant attempt at establishing synaptic interactions (evident in the elaboration of >> normal terminal projections and formation of tangles of neurotic processes within the target fields) the axons retract, the projections withdraw and the neurons die.

The analysis to date suggests that all CNS and PNS neurons that normally express CRD-NRG display this same pattern of initial extension, excessive axonal branching, withdrawal and death in the crd-nrg (-/-) animals.

Among the regions that express crd-nrg are all areas that are adversely affected in several neurodegenerative diseases including Alzheimers and Parkinsons disease.

Notable examples of crd-nrg expressing neurons include: projection neurons of the olfactory bulb, cholinergic projection neurons to cortex, thalamus, hippocampus and brainstem regions, dopaminergic and serotonergic neurons

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of brainstem, and cranial motor and sensory nuclei.

5 The formation of synapses is associated with a marked increase in the extent of nrg cyt-a translocation. The translocation of nrg cyt-a is induced by depolarization and this effect requires calcium entry.

10 Activation of nrg cyt a cleavage and nuclear translocation is co-ordinately controlled by nrg-erb interactions and electrical activity.

15 Disruption of this nrg mediated "back signaling" of transcription disrupts the normal pattern of neural connectivity, evident first in aberrant and excessive branching of crd-nrg + neuronal projections and the formation of neuritic tangles and, ultimately in neural degeneration and death.

20 The present invention is illustrated in the Experimental Details section which follows. This section is set forth to aid in an understanding of the invention but is not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

25

#### EXPERIMENTAL DETAILS

##### Example 1: Nuclear Signaling by the Cytoplasmic Domain of NRG-1.

30 Transmembrane neuregulin-1s (NRG-1) include an extracellular domain that is a ligand for erbB receptors

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and a highly conserved cytoplasmic domain of critical but unknown function. In this report we demonstrate that the cytoplasmic domain of NRG-1 translocates to the nucleus and regulates gene expression in neurons. Nuclear translocation is induced either following interaction of the NRG-1 extracellular domain with erbB receptors or following membrane depolarization. Nuclear targeting requires the first eight intracellular amino acids immediately following the transmembrane domain. Thus transmembrane isoforms of NRG-1 act not only as growth factors but also as bi-directional signaling molecules.

Many isoforms of the neuregulin-1 gene (NRG-1) are membrane anchored growth factors consisting of an extracellular domain containing the ligand, a single transmembrane domain and a highly conserved cytoplasmic domain (1, 2). Interactions between the extracellular domain of NRG-1 and erbB receptor tyrosine kinases have been studied extensively (3, 4). In contrast the possible functions of the large and highly conserved cytoplasmic domains of NRG-1 are less clear. Recently we demonstrated that neuronal NRG-1s that contain a cysteine rich extracellular domain (CRD-NRG-1) are required for the formation and maintenance of functional synapses (5). A striking feature of the phenotype of CRD-NRG-1<sup>-/-</sup> mice is the progressive loss of the neurons that would normally express the CRD-NRG-1 growth factor, but not the cells that express the erbB receptors. Mice that lack expression of all NRG-1 isoforms, all Ig-containing NRG-1 isoforms or all cytoplasmic domain containing NRG-1 isoforms die at E10.5, due to defects in the developing heart (6-8). This early death prevents detailed analyses of the effects of these mutations on NRG-1 expressing

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cells and their presumptive targets. However, even in these mutant embryos defects were observed in NRG-1 expressing neurons. These observations raise the possibility that membrane anchored NRG-1, in particular  
5 CRD-NRG-1, mediates bi-directional signaling, acting both as a ligand and a receptor for erbB tyrosine kinases. The proposed "back-signaling" in which CRD-NRG-1 acts as a receptor, is likely to be mediated by the NRG-1 cytoplasmic domain (NRG-1-CD). Additional lines of  
10 evidence supporting a function for the NRG-1-CD include the observation that ectopic expression of NRG-1 leads to NRG-1-CD dependent apoptosis (9, 10), and that the NRG-1-CD domain forms specific complexes with each other (11) or with other proteins, including LIM kinase (12), and a  
15 RING-finger protein of undetermined function (13). Here, we demonstrate that in neurons and in cultured cells the NRG-1-CD is in the nucleus, and we show that interaction with erbB2 and erbB4 receptors or membrane depolarization induce proteolysis of the transmembrane NRG-1, increase  
20 nuclear targeting of the NRG-1-CD and alter the expression of apoptotic genes.

Spiral ganglion neurons (SGNs) and hippocampal neurons express a high level of NRG-1 (14, 15). These neurons  
25 were isolated from E13.5 mouse embryos, grown in culture for 1-3 days, fixed and stained with antibodies specific for the NRG-1-CD (16) (the NRG-1c domain in Fig. 1A, the NRG-1a domain in Fig. 1B, 1C and 1E). Cultures were co-stained with antibodies recognizing either MAP-2 (Fig.  
30 1A) or neurofilaments (Fig. 1B, C and E). NRG-1-CD staining was seen in cell processes, cell bodies and in nuclei. Non-neuronal cells in these cultures did not stain with NRG-1-CD antibodies. Similar results were seen

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in both low-density (Fig. 1A) and high-density (Fig. 1B and C) neuronal cultures. In nuclei stained by the NRG-1-CD antibodies, a punctate staining pattern was typically seen (Fig. 1A, 1B & 1C) indicating that the NRG-1-CD was localized to sub-regions of these nuclei. About 15-20% of neuronal nuclei were positive for NRG-1-CD staining.

The ability of NRG-1-CD to translocate to nuclei makes it a strong candidate as a "reverse signal" implicated in the survival of motor and sensory neurons. In CRD-NRG-1<sup>-/-</sup> mice, neuron loss occurs after motor and sensory neurons enter target fields and initiate contacts that would normally result in synapse formation (5). In this light, it is possible that reverse signaling is initiated by NRG-1 interactions with erbBs on target cells, or by other signals (e.g. electrical activity) that occur following successful synaptogenesis. To investigate the role of NRG-1:erbB interactions and electrical activity in NRG-1-CD nuclear localization, E13.5 SGNs were cultured and depolarized by 50 mM KCl or treated with soluble erbB2 and erbB4 (serbB2, serbB4) (At E13.5 SGNs start to innervate hair cells that express erbB2 and erbB4. 17, 18). After treatment the subcellular localization of the NRG-1-CD was determined either by immunofluorescence (Fig. 1C and 1E) or immunoblotting (Fig. 1F). In untreated (Control) cultures, or in cultures treated with serbB2 (which does not directly interact with NRG-1) 15-20% of nuclei were positive for punctate NRG-1-CD staining. Following treatment with serbB2 and serbB4 or following treatment with 50 mM KCl for 15 min, >85% of nuclei stained positive for NRG-1-CD (Fig. 1D). The increase in punctate staining was

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accompanied by an increase in the level of a ~50 kD NRG-1-CD containing protein in the nuclei of serbB2/serbB4 or KCl but not serbB2 treated cells (Fig. 1F). Although it is not possible to demonstrate that the truncated product that appeared in nuclei was derived from a transmembrane precursor found in neuronal processes, there was a concomitant decrease in NRG-1-CD immunoreactivity in processes. This was most pronounced following KCl treatment (Fig. 1E) but was also seen in soluble erbB2 and erbB4 treated cultures (not shown).

Since NRG-1 only had been reported in membranous structures (plasma membrane, Golgi and endoplasmic reticulum) (19, 20), the presence of immunoreactivity in nuclei was unexpected. To confirm that NRG-1-CD was present in a functional nuclear compartment, and to determine whether stimuli that increase NRG-1 cleavage also increase nuclear translocation of NRG-1-CD, we expressed a series of chimeric NRG-1s in HEK 293T cells (Fig. 2A). The first chimera has a synthetic Gal4-VP16 transcription factor fused to the C-terminus of NRG-1Ba. HEK 293T cells were transfected with plasmids expressing either NRG-1Ba-Gal4-VP16 or Gal4-VP16 along with either a chloramphenicol acetyltransferase (pCAT) or luciferase (pLuc) reporter plasmids containing 4 copies of the Gal4 recognition sequence. Total chloramphenicol acetyltransferase (CAT) (Fig. 2B) or luciferase (Fig. 2C) activities were measured 48 hrs later. There was weak basal reporter gene expression in the absence of either Gal4-VP16 or NRG-1Ba-Gal4-VP16. Expression of Gal4-VP16 induced reporter gene expression about 6 fold. Expression of NRG-1Ba-Gal4-VP16 increased reporter gene expression 1.5 fold. Treatment of the transfected cells

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with phorbol-12-myristate-13-acetate (PMA) further increased reporter gene expression to a level 2.5 fold higher than control. This latter treatment is known to increase the cleavage of NRG-1 in its extracellular, juxtamembrane domain (19, 21). The effect of PMA on reporter gene expression is consistent with nuclear targeting of the NRG-1-CD following proteolytic cleavage of the full length, transmembrane form of NRG-1. The ability of the chimeric NRG-Gal4-VP16 protein to transactivate the target constructs indicates that at least this form of the NRG-1-CD is present in a transcriptionally active region of the nucleus.

To confirm that PMA induced cleavage of the transmembrane NRG and subsequent nuclear targeting of NRG-CD, and to rule out the possibility that nuclear translocation was orchestrated by sequences present in the Gal4-VP16 domain, we transfected cells with a vector encoding a transmembrane NRG with either a c-myc or HA epitope (Fig. 2D, F & G) or green fluorescent protein (GFP, Fig. 2E) fused to its C-terminus. HEK293T cells were transfected and visualized by immunofluorescence (Fig. 2D) or direct fluorescence (Fig. 2E) both before and after PMA treatment. Transfected cells stained with an anti-myc antibody showed perinuclear and faint membrane staining prior to PMA and both perinuclear/membrane and nuclear staining after PMA. Visualization of NRG-1-GFP in live cells allowed us to demonstrate clearly that PMA induced translocation of the fusion protein from a perinuclear, Golgi-like structure to the nucleus within 12 min (compare overlays of the 0 min and 12 min images in Fig. 2E). To determine the size of NRG-1-CD present in nuclei, we prepared nuclear extracts from HEK293T cells



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transfected with either the NRG-1B-Myc or NRG-1B-HA fusion protein and detected the NRG-1-CD by probing immunoblots with anti-myc or anti-HA antibodies. A faint band of about 50 kD was detected in nuclear fractions from transfected cells (Fig. 2F). The amount of this protein increased dramatically following PMA treatment (Fig. 2G). In contrast the particulate/membrane fraction contained proteins above 110 and 80 kDs (Fig 2F, lane 1), which we believe are the full length NRG-1 and a cleaved form lacking the erbB ligand binding domain, respectively.

The PMA-induced targeting of NRG-1-GFP to the nucleus indicates that following cleavage in the juxtamembrane region of NRG-1, the cytoplasmic domain is targeted to the nucleus. If this occurs then we predict that a truncated NRG-1-CD lacking extracellular and transmembrane sequences would be constitutively localized to the nucleus and further that the NRG-1-CD might contain an identifiable nuclear localization sequence (NLS). The first prediction was confirmed by expressing an NRG-1-CD-GFP chimera in which the NRG-1 coding sequences begin with the first amino acid following the transmembrane domain. Although green fluorescence was seen throughout cells expressing this chimera, there was a clear, very strong and punctate concentration of the fluorescent signal in nuclei (Fig. 3; note that the signal is so strong that it is visible in the blue channel used to visualize DAPI stained nuclei). Examination of the amino acid sequence of NRG-1-CDs identified 2 potential regions involved in nuclear localization (<http://psort.nibb.ac.jp>). One, NLS-1, included the first 8 amino acids following the transmembrane domain and is found in all NRG-1-CDs

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(KTKKQRKK). Deletion of these 8 amino acids (NRG-1bc-CD<sub>DNLS1</sub>-GFP) eliminated the strong nuclear staining seen when NRG-1bc-CD-GFP was expressed. This indicates that these 8 amino acids are required for nuclear localization of NRG-1-CD. The second, NLS-2 (PRLREKK), is present only in the a isoforms of NRG-1 and is not present in NRG-1bc-CD isoforms which appear in nuclei. When sequences unique to the NRG-1a-CD were fused to GFP, either with or without this NLS-2, no specific subcellular localization was observed (i.e. the pattern of green fluorescence was indistinguishable from that seen when GFP was expressed alone; data not shown).

The ability of specific signals to stimulate the cleavage and nuclear translocation of NRG-1-CD is reminiscent of signaling by the Notch and SREBP transcription factors (22-25). We used a DNA microarray to determine if changes in gene expression accompanied nuclear translocation of NRG-1-CD (26). Total RNA isolated from cultures of E13.5 SGNs that were either untreated or treated for 2 hrs with serbB2 and serbB4 were used as templates for synthesis of cDNA probes. These probes were then hybridized to mouse Atlas cDNA arrays. Clear differences in hybridization intensity were seen for at least 9 cDNAs. Expression of four mRNAs, MMCP-4, Oct-3, p19<sup>INK4</sup> and IL-11 increased whereas expression of five mRNAs, Bcl-X<sub>L</sub>, BAK, RIP, DP5 and Flt-3 decreased following treatment with serbBs. Changes in mRNA levels following treatment with serbB2 and serbB4 was confirmed by RT-PCR analysis of RNA isolated from additional cultures of SGNs (Fig. 4A) (27). Expression of Bcl-X<sub>L</sub>, BAK and RIP were repressed following treatment with soluble erbBs as well as following depolarization with KCl. Expression of DP5,

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Flt-3, and MMCP-4 was not consistently observed under any conditions using RT-PCR (data not shown). In addition we confirmed the up-regulation of Oct-3, p19<sup>INK4</sup> and IL-11 by RT-PCR following treatment with serbB2 and serbB4. In contrast, nuclear translocation of NRG-1-CD mediated by KCl treatment only induced expression of Oct-3 but not p19<sup>INK4</sup> or IL-11. To confirm that the effects on gene expression seen following treatment of SGN cultures with serbB2 and serbB4 resulted from interactions between these receptors and the extracellular domain of endogenously expressed NRG-1, we repeated these experiments in the presence of conditioned media containing the extracellular, erbB-binding domain of CRD-NRG-1 (Fig. 4B). SGN cultures were either untreated (Fig. 4B lanes 1) or treated with extracellular CRD-NRG-1 (Fig. 4B lanes 2), extracellular CRD-NRG-1 and serbB2/erbB4 (Fig. 4B lanes 3) or with serbB2 and serbB4 (Fig. 4B lanes 4). RT-PCR analysis of total RNA isolated from these cultures demonstrated that the repression of Bcl-X<sub>L</sub>, BAK and RIP expression by serbB2/serbB4 was competed successfully by the extracellular domain of CRD-NRG-1. Similarly the induction of both Oct-3 and p19<sup>INK4</sup> were blocked by co-treatment with the extracellular domain of CRD-NRG-1.

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We have demonstrated that NRG-1 is a bi-directional signaling molecule. Interaction with NRG-1 receptor results in well characterized signaling in erbB receptor expressing target cells. In addition interactions with these receptors results in nuclear translocation of the NRG-1 cytoplasmic domain. Although it is not known whether the NRG-1-CD binds to DNA or has a functional transcriptional activation domain, specific changes in

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gene expression occur following entry of NRG-1-CD into the nucleus. It is striking that in our original screen 4 of the 6 genes whose mRNA levels reproducibly changed encode products involved in either apoptosis or cell cycle progression. These results provide a clear connection between the response of NRG-1 expressing neurons to erbB receptors and the loss motor and sensory neurons that normally express CRD-NRG-1 in CRD-NRG-1<sup>-/-</sup> mutant mice.

#### Example 2: Novel Functions of the Cytoplasmic Domain of Neuregulin

Neuregulins (NRGs) comprise a large family of EGF-like growth factors expressed in both the CNS and PNS. The NRG 1 gene encodes multiple splice variants including secreted and transmembrane isoforms. The external (N-terminal) portion of both membrane anchored and secreted NRG isoforms includes a characteristic Ig-like or cysteine rich domain, and an EGF-like domain that is essential for NRG-ErbB interactions. Membrane anchored NRG isoforms identified to date include one of three distinct (a,b or c-type) cytoplasmic domains. Although the cytoplasmic domains are highly conserved (85% identity from chick to human), the biological function is unknown.

We tested whether NRG cytoplasmic domain(s) might mediate "back signalling" in NRG expressing cells, following interaction of the tethered ligand with erbB receptors. An intense subnuclear localization was observed in cells transfected with the NRG cytoplasmic domain. A similar

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observation was made in primary neuron immunostained with an antibody against the cytoplasmic tail of NRG. This nuclear translocation requires a NLS motif at the beginning of the cytoplasmic domain, which includes eight amino acids: KTKKQRKK. The motif is highly conserved (the same sequence of human, rat, mouse, chick and Xenopus NRG). The nuclear translocation was augmented by activation of PKC, or cells expressing erbB receptors. One of the functions of this nuclear translocation was to induce apoptosis. Furthermore, a novel gene (CNIP) was found to bind to the cytoplasmic domain of NRG, which might be the modulator for the functions of NRG cytoplasmic domain.

Example 3: Nicotinic receptors (nAChRs) participate in moving muscles, making memories, and reinforcing our most fundamental behaviors. CNS nAChRs are implicated in normal cognitive functions and their loss may underlie memory deficits associated with central cholinergic neurodegeneration. Stimulation of cholinergic projections or application of nicotine directly excites neurons by gating postsynaptic nAChRs and indirectly alters excitability by activation of presynaptic nAChRs, thereby enhancing transmitter release. Despite the key role of nAChRs in synaptic "tuning" and transmission, little is known of the regulatory mechanisms responsible for the expression, biophysical profile or cellular targeting of nAChRs in the CNS. We examine the cellular and molecular mechanisms underlying the regulation of nAChR expression at specific CNS synapses. We test whether interactions between pre and post synaptic partners control nAChR expression and the maturation of cholinergic sites in the CNS. The role of synaptic

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interactions in the expression and targeting of nAChRs to presynaptic sites are assayed in combined biophysical and molecular biological studies of nAChRs in visceral motor (VMN) and medial habenula (MHN) neurons before and after synapse formation. Parallel studies of input induced changes in post-synaptic nAChRs compare the expression profile and properties of nAChRs in interpeduncular (IPN) and amygdala neurons before and after synaptogenesis. We next test the hypothesis that cysteine-rich-domain neuregulins (CRD-NRG) are required for the synaptic differentiation of cholinceptive neurons in the CNS. Prior studies show that input-derived CRD-NRG controls nAChR expression in chick PNS and that CRD-NRG and NRG receptors (erbBs) are expressed by cholinergic and cholinceptive neurons in chick and mouse. To determine the role of CRD-NRG in mammalian synaptogenesis we generated a CRD-NRG "knock-out" mouse. Experiments assess the expression of CRD-NRG, erbBs and nAChRs in developing cholinceptive neurons within MHN, IPN and amygdala. If CRD-NRG is required for maturation of CNS cholinceptive synapses, as it is in the periphery, one can expect significant perturbations of the normal profile of expression and cellular targeting of nAChR channels to pre and post synaptic sites in CRD-NRG (-/-) mice. A final, long range, goal is to determine the signaling cascades activated by CRD-NRG. These studies pursue initial findings that both anterograde and retrograde signaling cascades are activated by CRD-NRG/erbB interactions.

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Decline in the levels of functional nAChRs and deterioration of central cholinergic projections have been implicated in aging-related memory deficits.

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Dramatic reductions in nAChRs and cholinergic neurons parallel the devastation of cognitive function in Alzheimer's disease. (Citations omitted). Deciphering the potential role of nAChRs in memory formation and the impact of deficits in nAChRs on cognition requires fundamental understanding of the mechanisms controlling the functional properties and cellular targeting of these receptors.

Stimulation of cholinergic projections or application of nicotinic agonists elicits fast excitatory currents via *postsynaptic* nAChRs. In addition, the activation of *presynaptic* nAChRs enhances synaptic transmission by increasing transmitter release. (Citations omitted).

Despite the numerous CNS relays shown to be subject to "synaptic tuning" by nAChR activation, neither the developmental changes nor regulatory signals that control the expression, biophysical profile or targeting of CNS nAChRs have been well understood. Our studies previously supported the identification, cloning and initial studies of a molecular signal that we now know is essential for the regulated expression of nAChRs in peripheral ganglia. We examine the cellular and molecular mechanisms controlling the expression and functional profile of CNS nAChRs, by addressing the following questions:

1. Do Neuron-neuron Interactions Regulate the Expression, Functional Profile and Cellular Distribution of CNS nAChRs?

Presynaptic input and target contact, coordinately regulate the profile and pattern of nAChRs expressed at

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developing ganglionic synapses in chick. (Citations omitted). The elaboration of nAChRs at both pre and postsynaptic sites of cholinceptive synapses in the CNS is likely controlled by similar cellular interactions.

5 (Citations omitted). Our studies test whether both cholinergic projections and target interactions control the expression and cellular targeting of nAChRs in the CNS. We compare the biophysical properties and expression profile of nAChRs in pre-synaptic visceral

10 motor (VMN) and medial habenula (MHN) neurons before and after contacting their respective targets. Parallel studies examine how presynaptic input influences the maturation of nAChRs in the somata-dendritic and axonal domains of cholinceptive neurons within the

15 interpeduncular nucleus (IPN) and amygdala. The studies described hereinabove constitute the essential groundwork for those referred to hereinbelow by combining anatomical, biophysical and molecular biological assays of *in vivo* and *in vitro* preparations. These studies

20 determine how neuronal interactions regulate: (a) the overall levels of nAChR expression; (b) the profile of the nAChR subunits and channel subtypes expressed; and (c) the somata-dendritic vs. axonal distribution of nAChRs during CNS synaptogenesis.

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## 2: Is CRD-NRG Required For Synaptogenesis-induced Changes in CNS nAChRs?

Previous studies affirmed that "CRD" isoforms of neuregulin are essential regulators of nAChR expression

30 in chick PNS.<sup>215</sup> CRD-NRG is neural specific, abundant in pre-ganglionic (VMN) neurons and is required for the input-dependent regulation of postsynaptic nAChRs in developing sympathetic ganglia. We test the hypothesis



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that CRD-NRGs are requisite signals for the synaptic induction, maturation and sustained expression of nAChR channels at pre and postsynaptic cholinceptive sites in the CNS. CRD-NRG is the predominant neuregulin isoform in the developing CNS with strong expression in brainstem, motor nuclei, and in subsets of midbrain and basal forebrain cholinergic neurons. Our studies pursue initial indications that CRD-NRG signaling is fundamental to the establishment of cholinceptive synapses and in the maturation of pre and postsynaptic nAChRs. We test whether CRD-NRG *mimics* input or target-induced changes in nAChRs by treatment of "synaptically naive" neurons with recombinant CRD-NRG *in vitro*. We also determine whether CRD-NRG is *required* for the regulated expression and maturation of nAChR channels following the initial formation of synaptic connections. *In vitro* studies compare the expression, functional profile and distribution of nAChRs in synaptic co-cultures treated with control or antisense oligonucleotides targeted against CRD-NRG. Physiological studies of neurons from WT vs. CRD-NRG<sup>-/-</sup> mice further test if CRD-NRG signaling is essential for the expression of the mature array of nAChRs at cholinceptive synapses.

### 3: What Signaling Cascades Are Activated by CRD-NRG?

A long-range goal is to determine the biochemical mechanisms underlying CRD-NRG effects in the CNS. Preliminary studies indicate that CRD-NRG activates a diverse array of signaling cascades, involving both anterograde and retrograde signaling mechanisms. (Citations omitted). We first examine the signaling

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pathways and molecular mechanisms that underlie the anterograde effects of CRD-NRG on neuregulin receptor (erbB) expressing neurons. By extending preliminary findings these studies determine the time course, dose dependence and relative affinity of recombinant, soluble CRD-NRG activation of specific kinase activated transcriptional cascades. We next pursue findings consistent with retrograde signaling by membrane-tethered CRD-NRG. Preliminary studies include our identification of a NRG cytoplasmic domain interactor-protein ("CNIP") and the demonstration that cleavage of CRD-NRG results in nuclear targeting of the cytoplasmic domain. Potential mechanisms of retrograde signaling via CRD-NRG are tested in cells stably expressing variants of tagged-NRG cytoplasmic domains  $\pm$  CNIP.

We examine the role played by synaptic interactions and candidate signals in regulating the induction and maturation of CNS nAChRs. We identify the cellular and molecular determinants that underlie the development, early plasticity, maintenance, and eventual demise of cholinceptive synapses in the brain. The following is an outline of recent findings.

*Nicotinic receptors and cholinceptive synapses in CNS function and dysfunction.* Recent efforts to dissect the role of central cholinergic projections and nicotinic receptors (nAChRs) in neurodegenerative diseases have unearthed new and intriguing leads, yet these issues remain controversial. In balance, recent reports support the fundamental importance of central cholinergic synapses in short-term, associative and motivational

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memory. (Citations omitted). Several studies document loss of CNS nAChRs and support the potential for nicotine based therapies in Alzheimer's Disease (AD). (Citations omitted). Of particular interest are studies demonstrating a direct relationship between the severity of symptoms of AD or Parkinson's disease and the loss of cholinergic and cholinceptive phenotypes in amygdala, hippocampus and substantia nigra. (Citations omitted). Dramatic declines in nAChRs in cholinergic neurons of the pedunculo pontine and medial septal nuclei are detected in AD specimen. (Citations omitted). A series of nAChRs assays in normal and AD patients led investigators to propose that quantitative assessment of nAChR down-regulation could be an early index of AD and PD neuropathology. (Citations omitted). Moreover, recent reviews suggest that changes in nAChRs expression and/or in nicotinic responses contribute to the symptoms, if not the etiology, of Alzheimer's Disease. (Citations omitted). Nevertheless, the importance of CNS nAChRs is not fully accepted. The conflict derives in part, from the classical bias that in the CNS, ACh alters excitability primarily via muscarinic, not nicotinic, receptors. The relative paucity of CNS nAChRs and limited demonstrations of direct, nAChR-mediated, synaptic transmission in the brain, continues to fuel the contention that nAChRs play little, if any, role at CNS cholinergic synapses.

Clinical relevance. In view of the emergent support for a fundamental role of cholinergic signaling in neurodegenerative diseases, we provide insight into the physiological and pathological regulation of central cholinceptive systems. Neither the cellular nor the

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molecular signals that control expression or function of CNS nAChRs are previously known. In fact the most basic experiments on CNS nAChR synaptic physiology have not previously been done. These basic data are required to confirm and extend prior anatomical and biochemical studies, and to provide the bases for understanding when and where nAChRs participate in cholinergic signaling in the CNS. Without these data, the role of nAChRs in CNS will remain a point of high controversy.

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*Activation of nAChRs on presynaptic terminals regulates the efficacy of synaptic transmission.* Classic studies of nicotine stimulated transmitter release from isolated nerve terminals, (citations omitted) and the (often ignored) work on nAChR mediated facilitation at ganglionic and neuromuscular synapses (citation omitted) have been widely confirmed. In fact, few neurotransmitters (including ACh) remain untouched by the nicotinic "tuning" of synaptic transmission. Multiple assays establish that activation of presynaptic nAChRs augments dopamine release in the ventral striatum. (citations omitted). The release of catechol- and indoleamines within numerous CNS nuclei is enhanced by nicotine. (citations omitted). GABAergic transmission is strongly potentiated by nAChR activation, an effect apparently involving both somata-dendritic and axon terminal nAChRs. (citations omitted). Last, but not least, electrophysiological studies in prefrontal cortex, interpeduncular nucleus and hippocampus document that even glutamate transmission is subject to control by cholinergic activation of presynaptic nAChRs (citations omitted).

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The parsimonious use of so few nAChR channels to control the gain of so many synaptic relays, suddenly clarifies how nicotine self-administration can exert such a diverse array of cognitive and behavioral effects. In view of the now established importance of pre-synaptic nAChRs in the CNS, it is essential to reconsider the role of nAChRs in CNS function and dysfunction in the more general context of "cholinoceptive sites" (whether pre or postsynaptic) rather than cholinergic synapses. In this view both pre and postsynaptic nAChRs are assessed as the substrates for the cholinergic control of synaptic plasticity. We evaluate how the number and the types of both pre and postsynaptic nAChR channels are regulated in the CNS.

*Neuronal nAChR complexes: Physiological properties and regulation of expression.* Understanding the role of nAChRs in the CNS requires (at least) a brief consideration of their diverse physiological properties. The family of neuronal nAChR subunit genes currently includes 11 members, designated as  $\alpha$ -type and  $\beta$ -type subunits. The 8  $\alpha$ -type nAChR subunits fall into two classes (citations omitted). One group, including  $\alpha 7, \alpha 8$ , and  $\alpha 9$ , are able to form functional, homomeric complexes that differ from heteromeric ( $\alpha/\beta$ ) nAChRs in sensitivity to nanomolar  $\alpha$ -bungarotoxin in (functional, homomeric complexes that differ from heteromeric ( $\alpha/\beta$ ) nAChRs in sensitivity to nanomolar ( $\alpha$ -bungarotoxin ( $\alpha$ BgTx)] or MLA. The remaining  $\alpha$ -subunits ( $\alpha 2$ - $\alpha 6$ ) require other  $\alpha$  and/or  $\beta$  subunits to form functional nAChRs. Expression of various combinations of  $\alpha$  and  $\beta$  subunits (i.e.  $\alpha_x/\alpha_y/\beta$  or  $\alpha/\beta_x/\beta_y$ ) yields biophysically and pharmacologically distinct nAChR channels. (citations omitted) Subtypes

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differ in their modulation by internal and external Ca, their distinct single channel kinetics ( $\gamma$ ), and in agonist and antagonist pharmacology. Neuronal nAChRs are more permeable to Ca than are muscle AChRs with  $\alpha 7$  homomers having the highest  $P_{Ca}/P_{Na}$  ( $>10$ ); Ca permeability also differs amongst other neuronal nAChRs (citations omitted). Inclusion of  $\alpha 5$  with other  $\alpha$ 's and yields complexes with  $>2$  x the Ca permeability of the comparable  $\alpha/\beta$  type complex. (citations omitted). These biophysical distinctions have direct impact on the efficacy of both presynaptic and postsynaptic nAChRs. High  $\gamma$ , brief  $\tau$  nAChRs are spatially segregated from low  $\gamma$ , long  $\tau$  channels at postsynaptic sites on sympathetic ganglion neurons: the synaptic currents that result are clearly distinct in amplitude and duration. Activation of  $\alpha 7$  containing presynaptic nAChRs enhances transmitter release at MHN-IPN synapses for up to 1 hour, whereas presynaptic  $\alpha/\beta$ -type nAChRs elicit more transient synaptic facilitation at interneuronal IPN synapses.

The numbers, functions and spatial distribution of nAChRs are highly regulated in developing PNS neurons. (citations omitted). Increased subunit nAChR mRNAs, increased macroscopic nAChR  $\gamma$ , increased number and probability of nAChR channel openings, and increased number of ligand-binding or immunoreactive sites have been reported. Preliminary work and recent studies of developing CNS reveal regional differences and subunit specific changes in nAChR expression concomitant with synaptogenesis. (citations omitted).

*Cholinoceptive sites in the Amygdala.*

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The amygdala is an integral component of the neural circuits important in memory. Selected regions of the amygdala are ravaged in AD, including the subregions that express nAChRs. (citations omitted). The basolateral (BL) and lateral olfactory tract (LOT) nuclei are the most prominent sites where nAChR mRNA and high affinity nicotine-and  $\alpha$ BgTx-binding are detected, and these nuclei are the principal recipients of cholinergic input. Shockingly, neither direct nAChR-mediated synaptic transmission nor nicotine-induced synaptic facilitation has been documented in the amygdala. Our preliminary studies reveal robust nicotine-induced enhancement of transmission at intra-amygdala synapses and suggest that presynaptic nAChRs are induced by a neuron-derived, regulatory signal (CRD-NRG; see below). The initial induction and maturation of nAChRs by amygdala neurons is examined.

*Cholinoceptive sites at Medial habenula-Interpeduncular synapses.*

The interpeduncular nucleus (IPN) receives the most robust cholinergic input of any subcortical structure in the brain. (citations omitted). Afferents include the MHN, medial septal cholinergic neurons, the vertical limb of the diagonal band, and the laterodorsal/pedunculopontine nuclear groups. Cholinergic terminals converge on IPN dendrites immediately apposed to other non-cholinergic synaptic boutons, consistent with the renowned effects of nicotine in modulating GABAergic and glutamatergic transmission in the IPN. (citations omitted) Although nicotine elicits direct (somatic) responses in IPN neurons, nAChR-mediated synaptic transmission is not detected in IPN slice or in

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MHN-IPN co-cultures. (citations omitted). Nevertheless, as the hub of CNS cholinergic projections and as a critical relay in higher sensory, arousal and reinforcement circuits, the IPN is an important site for testing mechanisms of nAChR regulation. Preliminary studies reveal that innervation increases expression and somata-dendritic nAChR responses. Furthermore, CRD-NRG (expressed by many, if not all, afferents to IPN) induces significant changes in nAChR expression. We examine pre and postsynaptic nAChRs at MHN-IPN synapses in detail.

*Neuregulin / erbB interactions in neural development and synaptic maturation.*

The neuregulin 1 (Nrg-1) gene encodes ligands for erbB tyrosine kinases. Differential splicing of primary Nrg-1 transcripts results in at least 15 distinct protein encoding mRNAs. Each of these isoforms is expressed in a unique temporal and tissue specific pattern. Specific NRG isoforms are implicated in neural and glial development and migration. Of particular import, specific isoforms NRG are required for the input-dependent induction of neuronal transmitter receptors for glutamate and ACh. We test whether CRD-NRG is essential for the initial induction, maturation and targeting of pre and postsynaptic nAChRs in the CNS. We initiate studies of NRG-signaling in cholinceptive neurons. Hence, a brief overview of NRG/erbB interactions. All NRGs contain the "EGF-like" domain required for receptor binding. Most isoforms are synthesized as single transmembrane domain proteins with one of three cytoplasmic domains. Soluble NRGs, generated by proteolytic cleavage in the external



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juxtamembrane domain, are thought to act in a paracrine or autocrine manner. NRGs activate erbB dimers, inducing extensive cytoplasmic domain tyrosine autophosphorylation. The resultant phosphotyrosines (p-Tyr) are bound by numerous signaling proteins containing either SH2 or PTB domains. These SH2/PTB containing proteins in turn activate specific signaling cascades, most of which involve serine/threonine protein kinases that phosphorylate targets in the plasma membrane, cytosol and nucleus of stimulated cells. The exact nature of the signaling complex assembled, and consequently the biological response, is dictated by the particular Tyr residues phosphorylated.

We identified a novel transmembrane NRG isoform that accounts for the presynaptic, nAChR-inducing activity in chick PNS. This NRG isoform is a novel variant containing a highly conserved (92% aa identity, chick vs. human) Cysteine Rich Domain N-terminal to the EGF domain. Disruption of the mouse Nrg-1 gene results in early embryonic lethality due to defective cardiac development, providing no information on the role of CRD-NRG in the maturation of central cholinceptive synapses. We have generated a CRD-NRG specific "knock out" mouse which survives until birth, consistent with CRD-NRGs exclusive expression in the nervous system. In vitro studies comparing different NRGs indicate that they have distinct biological effects, but do not relate these differences to specific downstream signaling. We begin to address this question in our experiments above. CRD-NRG transcripts encode transmembrane proteins. Recent data indicates that CRD-NRG acts as both ligand and receptor. Specific cytoplasmic domain interactor proteins have been

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identified.

Methods of making chimeric constructs are well known in the art. U.S. Pat. No. 4,237,224, the contents of which  
5 applicants hereby incorporate by reference into this application describes plasmid vectors for introducing foreign DNA into unicellular organisms.

Methods of preparing various pharmaceutical compositions  
10 with a certain amount of active ingredient are known, or will be apparent in light of this disclosure, to those skilled in this art. For examples of methods of preparing pharmaceutical compositions, see Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton,  
15 Pa., 18th Edition (1990).

U.S. Patent Nos. 6,043,260 and 6,051,597, the contents of which are hereby incorporated by reference into this application, provide additional information relating to  
20 preparing and administering pharmaceutical compositions in the treatment of diseases or conditions.

As used herein, the terms "treating", "treatment", and "treat" include curative, preventative (e.g. prophylactic)  
25 and palliative treatment.

As used herein, the term "composition", as in pharmaceutical composition, is intended to encompass a product comprising the active ingredient(s) and the inert  
30 ingredient(s) that make up the carrier, as well as any product which results, directly or indirectly from combination, complexation, or aggregation of any two or

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more of the ingredients, or from dissociation of one or more of the ingredients, or from other types of reactions or interactions of one or more of the ingredients.

5 As used herein, "effective amount" refers to an amount which is capable of treating or preventing a plaque rupture or superficial erosion or treating or preventing or delaying the onset of a disease or disorder or other clinical event described herein, or preventing or  
10 delaying the onset of macrophage death. Accordingly, the effective amount will vary with the subject being treated, as well as the condition to be treated.

Exact dosage and dosing schedules for the administration  
15 of the compounds and compositions described hereinabove can be determined by a skilled physician.

As used herein, "pharmaceutically acceptable carrier" means that the carrier is compatible with the other  
20 ingredients of the formulation and is not deleterious to the recipient thereof, and encompasses any of the standard pharmaceutically accepted carriers.

"Fusion protein" is a protein resulting from the expression of at least two operatively-linked  
25 heterologous coding sequences.

A coding sequence is "operably linked to" another coding sequence when RNA polymerase will transcribe the two coding sequences into a single mRNA, which is then  
30 translated into a single polypeptide having amino acids derived from both coding sequences. The coding sequences need not be contiguous to one another so long as the

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expressed sequence is ultimately processed to produce the desire protein.

5 "Recombinant" polypeptides refer to polypeptides produced by recombinant DNA techniques; i.e., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide. "Synthetic" polypeptides are those prepared by chemical synthesis.

10 A "vector" is a replicon, such as a plasmid, phage, or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

15 A DNA or a "nucleotide sequence encoding" a particular protein, is a DNA sequence which is transcribed and translated into a polypeptide in vivo when placed under the control of appropriate regulatory sequences.

20 A "promoter sequence" or "promoter" or "promoter region" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence.

25 Other features and advantages of this invention will be apparent from the specification and claims which describe the invention.

30 Example 4: Back Signaling by the NRG-1 Intracellular Domain

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Transmembrane isoforms of neuregulin-1 (NRG-1), ligands for erbB receptors, include an extracellular domain with an EGF-like sequence and a highly conserved intracellular domain of unknown function. In this report we demonstrate that transmembrane isoforms NRG-1 are bi-directional signal molecules in neurons. The stimuli for NRG-1 back signaling include binding of ErbB receptor dimers to the extracellular domain of NRG-1 and neuronal depolarization. These stimuli elicit proteolytic release and translocation of the intracellular domain of NRG-1 to the nucleus. Once in the nucleus, the NRG-1 intracellular domain represses expression of several regulators of apoptosis, resulting in decreased neuronal cell death in vitro. Inhibiting  $\gamma$ -secretase activity blocks the pro-survival and transcriptional activity of NRG-1 and substantially increases total NRG-1 levels. Thus,  $\gamma$ -secretase dependent processing of NRG-1 results in retrograde signaling that appears to mediate contact and activity dependent survival of NRG-1 expressing neurons.

Many isoforms of the neuregulin-1 gene (NRG-1) are membrane anchored growth factors consisting of an extracellular domain, a single transmembrane domain and a highly conserved cytoplasmic domain (1, 2). Interactions between the extracellular domain of NRG-1 and erbB receptor tyrosine kinases have been studied extensively (3-5). In contrast the possible functions of the large and highly conserved intracellular domains of NRG-1 are less clear. Recently we demonstrated that neuronal NRG-1s that contain a cysteine rich

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extracellular domain (CRD-NRG-1) are required for the formation and maintenance of functional motor and sensory synapses (6). A striking feature of the phenotype of CRD-NRG-1<sup>-/-</sup> mice is the progressive loss of the motor and sensory neurons that would normally express the CRD-NRG-1 growth factor. In CRD-NRG-1<sup>-/-</sup> mice, neuron loss occurs after neurons enter target fields and initiate contacts that would normally result in synapse formation (6). In this light, it is possible that reverse signaling is initiated by NRG-1 interactions with erbBs on target cells, or by other signals (e.g. electrical activity) that occur following successful synaptogenesis. Mice that lack expression of all NRG-1 isoforms, die at E10.5, due to defects in the developing heart (7-9). This early death prevents detailed analyses of the effects of these mutations on other NRG-1 expressing cells and their presumptive targets. However, even in these mutant embryos defects were observed in NRG-1 expressing neurons. These observations raise the possibility that membrane anchored NRG-1, in particular CRD-NRG-1, mediates bi-directional signaling, acting both as a ligand and a receptor for erbB tyrosine kinases. The proposed "back-signaling" in which CRD-NRG-1 acts as a receptor, is likely to be mediated by the NRG-1 intracellular domain (NRG-1-ICD). Additional lines of evidence supporting a function for the NRG-1-ICD include the observation that ectopic expression of NRG-1 leads to NRG-1-ICD dependent apoptosis (10, 11), and that the NRG-1-ICD domain forms specific complexes with cytoplasmic proteins, including LIM kinase (12) and a RING-finger protein of undetermined function (13). Here, we demonstrate that in CRD-NRG-1 expressing neurons and in cell lines transfected with full length transmembrane

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CRD-NRG-1, interaction with erbB2 and erbB4 receptors induces proteolysis of the transmembrane NRG-1, increases nuclear targeting of the NRG-1-ICD, alters the expression of apoptotic genes and promotes survival of neurons in vitro.

Spiral ganglion neurons (SGNs) and hippocampal neurons express a high level of the CRD-NRG-1 isoform (14, 15). Neurons were isolated from E14 mouse embryos, maintained in vitro for 1-7 days and stained with antibodies specific for the longest NRG-1-ICD ("a-form") (16). Neurons were also visualized by staining with antibodies recognizing either NF-1 or MAP-2. Under control conditions immunoreactive NRG-1-ICD appeared diffusely distributed in most neuronal soma, along processes, and more rarely (in 15-20% of neurons), in a punctuate pattern in neuronal nuclei (Fig 8A and 8B). Non-neuronal cells in these cultures did not stain with NRG-1-ICD antibodies and staining was completely blocked by preincubation of this antibody with the C-terminal peptide of ICD (data not shown).

The presence of NRG-1-ICD in neuronal nuclei raises the possibility that nuclear targeting of the NRG-1-ICD might participate in the back signaling implicated in the survival of motor and sensory neurons. To investigate the role of NRG-1:erbB interactions and electrical activity in NRG-1-ICD nuclear localization, primary spiral ganglia neurons were treated with soluble erbB2 and erbB4 (erbB2:B4) or depolarized by 50 mM KCl (17). In untreated (Control) cultures, 15-20% of nuclei were positive for NRG-1-ICD staining. After fifteen minutes of treatment with soluble erbB2:B4 or K<sup>+</sup> depolarization, >85% of nuclei stained positive for ICD (Fig. 8A-C).

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Immunoblots of SGN cytoplasmic and nuclear extracts (18) probed with the NRG-1-ICD specific antibody revealed two specific bands: a major protein of ~110kD and a minor protein of ~50kD (Fig. 8D). These two proteins appear to correspond to the proposed precursor form of NRG-1 and a processed form containing the ICD, respectively. The erbB2:B4 and KCI induced increases in nuclear staining were accompanied by increases in the level of NRG-1-ICD in the nuclear fraction of serbB2:B4 or KCI, but not serbB2 treated cells (Fig. 8D and 8E).

To gain more insight into the dynamics of regulated nuclear targeting of the NRG-ICD, we expressed a series of chimeric CRD-NRG-1s in HEK 293T cells (Fig. 9A) (19). Subcellular targeting of NRG-1 was followed in living cells transfected with a CRD-NRG-1 $\beta$ a-GFP fusion protein by collecting images every two minutes with a two-photon microscope (Fig. 9B). In control cells, most of the CRD-NRG-1-GFP was concentrated around the cell periphery and in a single intracellular region, consistent with previous reports of NRG-1 localization in the plasma membrane, Golgi and endoplasmic reticulum (20, 21). This pattern remained essentially unchanged for up to 2 hours of continuous observation. Two to four minutes after treatment with serbB2:B4, distinct fluorescent aggregates were seen both in peripheral regions and near the Golgi-like structure. These aggregates appeared to move along discrete paths and by 10 minutes punctate patches of fluorescent signal were observed in the nucleus (Fig. 9B). Cytoplasmic and nuclear extracts from HEK293T cells expressing a CRD-NRG-1 $\beta$ a-HA fusion protein (full length NRG-1 $\beta$ a tagged at the C-terminus with an 11 amino acid HA



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epitope) were prepared and the NRG-1-ICD was detected by probing immunoblots with an anti-HA antibody. In control (Fig. 9C; serbB2) the ~110 kD full-length protein and several higher molecular weight bands were detected. These higher molecular weight bands likely correspond highly glycosylated or possible aggregated forms of NRG-1. Treatment of transfected cells with soluble erbB2 and erbB4 resulted in increased amounts of a ~50 kD protein corresponding to the NRG-1-CD (Fig. 9C; serbB2:B4). In contrast to the case with NRG-1 expressed in neurons, there was a significant amount of apparently full length NRG-1 associated with the nuclear fractions of transfected cells. The significance of this is not clear, but this form does not appear to be active in back-signaling since inhibition of  $\gamma$ -secretase significantly increased the amount of this protein in all subcellular fractions but this form does not appear to be active in back-signaling since inhibition of  $\gamma$ -secretase significantly increased the amount of this protein in all subcellular fractions but inhibited all measurable back signaling (see below).

Nuclear targeting of NRG-1-ICD indicates that NRG-1 might possess nuclear localization motifs (NLS). Examination of the amino acid sequence of NRG-1 identified 2 potential NLS motifs. One included the first 8 intracellular amino acids following the transmembrane domain and is found in all NRG-1 ICDs (KTKKQK), whereas the second (PRLREKK), is present only in "a" isoforms. Deletion of the first motif, but not the second, prevented nuclear targeting of ICD-GFP fusion proteins in transfected cells, supporting a role for this motif in

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nuclear localization following release of the NRG-1 ICD from the membrane (data not shown).

To test whether the NRG-1-ICD not only translocates into  
5 nuclei, but has transcriptional activity, we measured the ability of a fusion protein of the ICD and the yeast Gal 4-DNA binding domain (ICD-Gal4<sub>DBD</sub>) to activate a Gal4 UAS-luciferase reporter plasmid (22). HEK 293T cells were co-transfected with plasmids expressing either NRG-1 $\beta$ -  
10 Gal4-VP16 (the full length, trans-membrane NRG-1 fused to a Gal4-VP16 synthetic transcription factor) or ICD-Gal4 with the Gal4-UAS-luciferase reporter plasmid. Total luciferase activity was measured 48 hrs later (Fig. 9D). Expression of the non-membrane tethered ICD-Gal4<sub>DBD</sub>  
15 dramatically increased luciferase activity, about 10-fold compared to the full-length NRG-1 fused to Gal4-VP16. Since the Gal4<sub>DBD</sub> lacks a transactivation domain, expression of luciferase indicates that the ICD has an intrinsic activation domain.

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The appearance of a ~50kD C-terminal fragment of NRG-1 in  
nuclei after serbB2:B4 or KCI treatment is consistent with regulated cleavage of the transmembrane precursor  
25 form of NRG-1 (Fig. 8D). Constitutive and regulated extracellular cleavage of both type 1 and type III NRG-1 has been characterized (23-25), but events leading to release of the NRG-1-ICD from the membrane have not been studied. Because the first 8 intracellular amino acids  
30 are required for nuclear translocation, the cleavage event that releases the ICD is expected to occur at the junction between this sequence and the transmembrane domain, or within the transmembrane domain. We tested

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whether  $\gamma$ -secretase, a protease known to catalyze intra-membranous proteolysis(26), was involved in NRG-1-ICD processing. HEK 293T cells expressing NRG-1-GFP were treated with a specific inhibitor of  $\gamma$ -secretase (MW-III-26A) for 8 hours prior to treatment with serbB2:B4. Localization of the GFP-tagged NRG-1-ICD in response to serbB2:B4 treatment was observed with a two-photon microscope (Fig. 10A). In the majority of transfected cells, pre-treatment with the  $\gamma$ -secretase inhibitor resulted in a dramatic increase in the fluorescence signal detected throughout the cell consistent with diffuse membrane and cytoplasmic localization of full length NRG-1-ICD-GFP. The increase in unprocessed NRG-1 that resulted from inhibiting  $\gamma$ -secretase was confirmed by immunoblotting (Fig. 10B). Treatment of transfected cells with  $\gamma$ -secretase inhibitors greatly increased steady state levels of full length NRG-1; proteolytic processing of NRG-1 could not be detected. As an additional test of the role of  $\gamma$ -secretase in regulated processing of NRG-1-ICD and consequent transcriptional activation, we co-transfected 293T cells with NRG-1 $\beta$ a-Gal4-VP16 and the Gal4-UAS-luciferase reporter plasmid (Fig. 10C). Treatment with serbB2:B4 heterodimers significantly elevated luciferase levels. This increase was partially blocked by pre-incubation of soluble erbB2:B4 with the receptor-binding, NRG-1 ECD domain, or by pretreating cells with inhibitors of  $\gamma$ -secretase. No inhibition was seen in cells pretreated with a closely related, but inactive analogue. Therefore, the stimulated nuclear targeting and transcriptional activity of the NRG-1-ICD is dependent on  $\gamma$ -secretase activity. This requirement is consistent with a model in which NRG-1:erbB interactions result in intra-membrane cleavage of

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NRG-1 and the subsequent release of the NRG-1-ICD.

The experimental results presented above demonstrate that translocation of NRG-1-ICD to the nucleus can regulate transcription. To assess whether stimulation of NRG-1-ICD regulates gene transcription in neurons total RNA isolated from neuronal cultures that were either untreated or treated for 2 hrs with serbB2:B4 were used to probe a mouse cDNA array (27). Clear differences in hybridization intensity were seen for at least 9 cDNAs; MMCP-4, Oct-3, p19<sup>INK4</sup>, IL-11, Bcl-XL, BAK, RIP, DP5 and Fit-3. Changes in mRNA levels following treatment with serbB2:B4 were confirmed by RT-PCR analysis of RNA isolated from additional neuronal cultures (Fig. 11A and 11B) (28). Expression of Bcl-X<sub>L</sub>, BAK and RIP, KCI only induced expression of Oct-3 but not p19<sup>INK4</sup> or IL-11. Thus, when induced by KCI nuclear translocation of NRG-1-ICD did not activate expression of these targets. Expression of DP5, Flt-3, and MMCP-4 was not consistently observed under any conditions using RT-PCR and therefore the effect of serbB2:B4 treatment could not be confirmed.

To confirm that the effects on gene expression seen following treatment of neuronal cultures with serbB2:B4 resulted from interactions between these receptors and the extracellular domain of endogenously expressed NRG-1, neuronal cultures were either untreated (Fig. 11B, lane 2), treated with serbB2:B4 (Fig. 11B lane 3) or treated with a mix of serbB2:B4 and the ECD (Fig. 11B, lane 4). RT-PCR analysis demonstrated that effects of serbB2:B4 were blocked by the NRG-1 ECD.

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The demonstration that nuclear translocation of the NRG-1-ICD resulted in changes in expression of apoptosis regulating genes, taken together with our previous demonstration that disrupting the CRD-NRG-1 gene in mice results in loss of neurons expressing this protein (6), led us to ask whether contact dependent targeting of the NRG-1-ICD to the nucleus could promote neuronal survival. Dispersed neurons were maintained in culture for 2 days and then they were treated overnight with combinations of serbB2:B4, soluble NRG-ECD or  $\gamma$ -secretase inhibitors. Apoptotic neurons were counted following staining of nuclei with bisbenzamide. In control cultures 20% of the neurons had the small condensed nuclei that characterize apoptosis (Fig. 11C and 11D). Treatment with serbB2:B4 reduced the number of apoptotic neurons by ~50%. This reduction was not seen if the serbB2:B4 was pre-incubated with soluble NRG-ECD or if cultures also were treated with  $\gamma$ -secretase inhibitor.

We have demonstrated that NRG-1 is a bi-directional signaling molecule (Fig. 12). Interaction of the NRG-1 ECD with erbB receptors (erbB3 and erbB4), results in well characterized signaling in the erbB expressing target cells. In neurons NRG-1 processing and NRG-1-ICD nuclear targeting are regulated by either interaction with erbB2:B4 or depolarization, and results in changes in gene expression. Stimulated, and possibly basal, nuclear translocation of NRG-1-ICD depends on  $\gamma$ -secretase activity. It is striking that in our original cDNA screen 4 of the 6 target genes encode products involved in either apoptosis or cell cycle progression, and that nuclear targeting of the NRG-1-ICD prevents neuronal apoptosis. Together these results support the conclusion that NRG-1-ICD back signaling provides a survival signal

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for maintaining synaptic interactions. This model could explain the loss of NRG expressing neurons that have been consistently reported in mice in which all, or part of the NRG-1 gene has been disrupted (6-9).

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The bi-directional role and the  $\gamma$ -secretase dependent processing of NRG-1 are reminiscent of several other proteins involved in neuronal development and function. What is apparently unique to NRG-1 is the combination of  
10 bi-directional signaling (similar to that reported for ephrin B) with the regulated release of a transcriptional regulator from a membrane tethered, inactive precursor (as reported for Notch, APP (SRE-BP) (26, 29-13)).

## 15 Reference

1.  
G.D. Fischbach, K.M. Rosen, *Annu. Rev. Neurosci.* **20**, 429 (1997).
2.  
20 M. Gassmann, G. Lemke, *curr. Opin. Neurobio.* **7**, 87 (1997).
3.  
A. Buonanno, G.D. Fischbach. *Curr. Opin. Neurobiol.* **11**, 287 (2001).
- 25 4. K.L. Carraway II, S.J. Burden, *Curr. Opin. Neurobiol.* **5**, 606 (1995).
5. S.J. Burden, Y. Yarden, *Neuron* **18**, 847 (1997).
6. D. Wolpowitx et al., *Neuron* **25**, 79 (2000).
7. D. Meyer, C. Birchmeier, *Nature* **378**, 386 (1995).
- 30 8. R. Kramer et al., *Proc. Natl. Acad. Sci. USA* **93**, 4833 (1996).
9. X. Liu et al., *Proc. Natl. Acad. Sci. USA* **95**, 3024 (1998).

10. S. Grimm, P. Leder, *J. Exp. Med.* 185, 1137 (1997).
11. S. Grimm, E.J. Weinstein, I.M. Krane, P. Leder, *J.*  
5 *Exp. Med.* 188, 1535 (1998).
12. J.Y. Wang, K.E. Frenzel, D. Wen, D.L. Falls, *J.*  
*Biol. Chem.* 273, 20525(1998).
13. J. Bao, D.A. Talmage, L.W. Role, *Society For*  
10 *Neuroscience Abstracts*, 562.1(1997).
14. B.J. Morley, *Brain Res. Mol. Brain Res.* 54,  
170(1998).
15. 15. M.S. Chen et al., *J. Comp. Neurol.* 349, 389(1994).
16. Primary neuronal cultures were fixed with 4%  
paraformaldehyde and 4% sucrose in PBS for 15  
minutes, and permeabilized with 0.25% Triton X-100  
20 in PBS for 5 minutes. The cells were washed three  
times in PBS and incubated in 10% normal goat serum  
for one hour at 37 °C. Cells were incubated  
overnight at 4 °C in a cocktail of rabbit polyclonal  
antibodies against NRG-1-ICD (1:100, sc-348 or sc-  
25 537, Santa Cruz Biotech.) and mouse monoclonal  
antibodies against neurofilaments 68 and 160 kD  
(1:2000, NCL-NF68 and NCL-NF160, Novocastra Lab.) or  
Map-2 (sc-5357, Santa Cruz Biotech.) in PBS with 3%  
normal goat serum. The cells were washed and  
30 incubated with rhodamine or FITC-conjugated  
secondary antibodies (1:1000, Jackson ImmunoResearch  
Lab.) and TOTO-3 (1 µM, Molecular Probes) for 1 hour  
at 37 °C. The cells were viewed with a confocal  
argon/krypton laser microscope. Data were collected

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with Carl Zeiss LSM software and analyzed as an overlay projection of 5 1- $\mu$ M sections through the beginning and the end of the nucleus with three different channels.

- 5 17. M.C. Whitehead, *Neurobiology of Hearing* (Raven Press, New York, 1986), pp. 190-198.
18. Cytoplasmic, particulate and nuclear fractions from transfected cells were prepared using "Nuclear and Cytoplasmic extraction reagents" (PIERCE). 200  $\mu$ l  
10 cytoplasmic fraction, 50  $\mu$ l particulate fraction and 100  $\mu$ l nuclear fraction were obtained from one 10 cm dish. Protein concentration from each sample were measured a Bio-Rad protein assay kit based on the Bradord method (Bio-Rad Lab.). 40  $\mu$ g nuclear, 40  $\mu$ g  
15 particulate and 120  $\mu$ g cytoplasmic protein were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes (Schleicher & Schuell), and probed with antibodies against NRG-1-ICD, histone H1 or eIF5. Molecular mass was estimatd by comparing  
20 the relative mobility of immunoreactive bands to prestained SDS-PAGE standards (Low Range, Bio-Rad).
19. Epitope-tagged full-length or truncated forms of NRG- $\beta$ 1a were prepared by the polymerase chain reaction (PCR), and cloned into pCDNA3.1/V5/His or pCDNA3.1/CT-GFP0TOPO (invitrogen). The primer pair  
25 for fusing full-length CRD-NRG-1 $\beta$ a with the HA epitope was: 5': ACAT GTCTG AGGGA GCTGG CGGGA GGT and 3' TCATA CAGCG TAGTC TGGGA CGTCG TATGG GTA. The PCR primer pair used to fuse full-length NRG-1 $\beta$ a with  
30 GFP was: 5': AGCAT GGCTG AGAAG AAGAA GGAAA AA and 3': TACAG CAATG GGGTC TTGAT TCGTT ATTAC ACT. The PCR primer pair used to fuse the cytoplasmic domain containing NLS-1 (aa 295-390) to GFP was: 5': ATTAT



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- GAAAA CCAAG AAACA GAGA and 3': GACCA TTACT CCAGC TGTGA CTTG. The PCR primer pair used to fuse the cytoplasmic domain lacking NLS-1 (aa 304-390) to GFP was 5': ATTAT GTTGA ATGAC CGTTT AAGA and 3': GACCA TTACT CCAGC TGTGA CTTG. DNA sequences were confirmed by dideoxynucleotide sequencing.
20. T.L. Burgess, S.L. Ross, Y. Qian, D. Brankow, S. Hu, *J. Biol. Chem.* **270**, 19188 (1995).
21. C. Rio, H.I. Rieff, P. Qi, G. Corfas, *Neuron* **19**, 39(1997).
22. p4luc (from R. Evans) which contains four copies of the Gal4-UAS element fused to the firefly luciferase coding region, was used as a reporter. The following plasmids were co-transfected into 293T cells in various combinations: 10  $\mu$ g p4luc, 20 $\mu$ g of NRG- $\beta$ 1a-Gal4-VP16 or 20 $\mu$ g NRG-1-ICD-Gal4<sub>DBD</sub> and 2  $\mu$ g of pcDNA-GFP (used to determine transfection efficiency). Total amounts of transfected DNA were kept constant by adding the appropriate amount of pcDNA. Lysates were prepared 48 hr after transfection for reporter expression assay. GAL4-VP16 was fused in frame to the carboxyl termini of full-length NRG-1 $\beta$ a, and was cloned by the PCR into pcDNAA3.1/V5/His-TOPO (Invitrogen). Primer pairs used for amplification of Gal4-VP16 were 5': GTATA CCCAT ACCCG CCGAA GCTT and 3': CTTAT ACTCC ACGT ACTCG TCAA; and for amplification of NRG- $\beta$ 1a: 5': ATGGC TGAGA AGAAG AAGGA AAAAG AA and 3': GTATG GGTAT ACAGC AATGG GGTCT TG. DNA sequences were confirmed by dideoxynucleotide sequencing.
23. J.A. Loeb, E.T. Susanto, G.D. Fischbach, *Mol. Cell, Neurosci.* **11**, 77(1998).
24. J.Y. Wang, S.J. Miller, D.L. Falls *J. Biol Chem.*

-77-

- 276, 2841 (2001).
25. X. Liu et al., *J. Biol. Chem.* 273, 34335 (1998).
26. M.S. Brown, J, Ye, R.B. Rawson, J.L. Goldstein, *Cell*, 100, 391 (2000).
- 5 27. Total RNA isolated from untreated and serbB2:B4 treated primary E13.5 SGN cultures was <sup>32</sup>P-labeled using the Atlas Pure Total RNA labeling systems (Clontech) and hybridized separately to the Atlas Mouse 1.2 Array containing 1,176 cDNAs (Clontech).
- 10 After a high-stringency wash and autoradiography, expression profiles between the two hybridization patterns were noted upon visual examination. We identified five genes down regulated (BcI-X<sub>L</sub>, BAK, RIP, DP5, and Flt-3), and four genes up-regulated
- 15 (MMCP-4, Oct-3, p19<sup>INK4</sup>, and IL-11) by serbB2:B4.
28. Total RNA from E13.5 SGN cultures was used for RT-PCR. PCR reactions were performed for 35 cycles (45 s at 94 °C, 60 s at 52 °C, and 90 s at 72 °C) in a volume of 25 µl containing 1 X PCR buffer, 100µM
- 20 dNTPs, 1µM each primer, and 1 U of Taq polymerase (Boehringer-Mannheim). Reactions were done in triplicate. Amplified products were separated on 3% NuSeive agarose gels and the band intensity was compared to amplified actin bands. Samples in
- 25 parallel but without reverse transcriptase added were used as negative controls.
29. G. Struhl, A. Adachi, *Cell* 15, 649 (1998).
30. S. Jeffries, A.J. Capobianco, *Mol. Cell Biol.* 20, 3928 (2000).
- 30 31. X. Cao, T. Sudhof, *Science* 293, 115 (2001).

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It should be noted that this invention is not limited to the particular embodiments described herein, but that various changes and modifications may be made without departing from the spirit and scope of this novel concept  
5 as defined by the claims which follow.

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References

1. G.D. Fischbach, K.M. Rosen, *Annu. Rev. Neurosci.* 20,  
429 (1997).
- 5 2. M. Gassmann, G. Lemke, *Curr. Opin. Neurobio.* 7, 87  
(1997).
3. K.L. Carraway III, S.J. Burden, *Curr. Opin. Neurobiol.*  
10 5, 606 (1995).
4. S.J. Burden, Y. Yarden, *Neuron* 18, 847 (1997).
5. D. Wolpowitz et al., *Neuron* 25, 79 (2000).
- 15 6. D. Meyer, C. Birchmeier, *Nature* 378, 386 (1995).
7. R. Kramer et al., *Proc. Natl. Acad. Sci. USA* 93, 4833  
(1996).
- 20 8. X. Liu et al., *Proc. Natl. Acad. Sci. USA* 95, 13024  
(1998).
9. S. Grimm, P. Leder, *J. Exp. Med.* 185, 1137 (1997).
- 25 10. S. Grimm, E.J. Weinstein, I.M. Krane, P. Leder, *J.*  
*Exp. Med.* 188, 1535 (1998).
11. X. Liu et al., *J. Biol. Chem.* 273, 34335 (1998).
- 30 12. J. Y. Wang, K.E. Frenzel, D. Wen, D.L. Falls, *J.*  
*Biol. Chem.* 273, 20525 (1998).

-80-

13. J. Bao, D.A. Talmage, L.W. Role, *Society For Neuroscience Abstracts*, 562.1 (1997).

14. B.J. Morley, *Brain Res. Mol. Brain Res.* 54, 170  
5 (1998).

15. M.S. Chen et al., *J. Comp. Neurol.* 349, 389 (1994).

16. Primary neuronal cultures were fixed with 4%  
10 paraformaldehyde and 4% sucrose in PBS for 15 minutes,  
and permeabilized with 0.25% Triton X-100 in PBS for 5  
minutes. The cells were washed three times in PBS and  
incubated in 10% normal goat serum for one hour at 37 °C.  
Cells were incubated overnight at 4 °C in a cocktail of  
15 rabbit polyclonal antibodies against NRG-CD (1:1000, sc-  
348 or sc-537, Santa Cruz Biotech.) and mouse monoclonal  
antibodies against neurofilaments 68 and 160 kD (1:2000,  
NCL-NF68 and NCL-NF160, Novocastra Lab.) or MAP-2 (sc-  
5357, Santa Cruz Biotech.) in PBS with 3% normal goat  
20 serum. The cells were washed and incubated with rhodamine  
or FITC- conjugated secondary antibodies (1:1000, Jackson  
ImmunoResearch Lab.) and TOTO-3 (1 mM, Molecular Probes)  
for 1 hour at 37 °C. The cells were viewed with a confocal  
argon/krypton laser and Carl Zeiss microscope. Data were  
25 collected with Carl Zeiss LSM software and analyzed as an  
overlay projection of 5 1-mm sections through the  
beginning and the end of the nucleus with three different  
channels.

30 17. M.C. Whitehead, *Neurobiology of Hearing* (Raven Press,  
New York, 1986), pp. 190-198.

18. J. Bao et al., unpublished data.

-81-

19. T.L. Burgess, S.L. Ross, Y. Qian, D. Brankow, S. Hu,  
*J. Biol. Chem.* 270, 19188 (1995).
20. C. Rio, H.I. Rieff, P. Qi, G. Corfas, *Neuron* 19, 39  
5 (1997).
21. J.A. Loeb, E.T. Susanto, G.D. Fischbach, *Mol. Cell.  
Neurosci.* 11, 77 (1998).
- 10 22. R.P. Brun, J.B. Kim, E. Hu, B.M. Spiegelman, *Curr.  
Opin. Lipidol.* 8, 212 (1997).
23. M.S. Brown, J. Ye, R.B. Rawson, *Cell* 100, 391 (2000).
- 15 24. G. Struhl, A. Adachi, *Cell* 15, 649 (1998).
25. S. Jeffries, A. J. Capobianco, *Mol. Cell Biol.* 20,  
3928 (2000).
- 20 26. Total RNA isolated from untreated and serbB 2/4  
treated primary E13.5 SGNs cultures was <sup>32</sup>P-labeled using  
the Atlas Pure Total RNA labeling Systems (Clontech) and  
hybridized separately to the Atlas Mouse 1.2 Array that  
contains 1,176 genes (Clontech). After a high-stringency  
25 wash and autoradiography, expression profiles were  
obtained for untreated and erbB 2/4 treated groups.  
Obvious differences between the two hybridization  
patterns were noted upon visual examination. We  
identified five genes (Bcl-X<sub>L</sub>, BAK, RIP, DP5, and Flt-3),  
30 down-regulated by serbB2 and serbB4 and four genes (MMCP-  
4, Oct-3, p19<sup>INK4</sup>, and IL-11) up-regulated by serbB2 and  
serbB4. RT-PCR was used subsequently to confirm changes  
in expression of Bcl-X<sub>L</sub>, BAK, RIP, DP5, Flt-3, MMCP-4,

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Oct-3, p19<sup>INK4</sup> and IL-11.

27. Total RNA from E13.5 SGNs cultures was used for RT-PCR. PCR reactions were performed for 35 cycles (45 s at 94 °C, 60 s at 52 °C, and 90 s at 72 °C) in a volume of 25 ml containing 1 X PCR buffer, 100 mM dNTPs, 1 mM each primer, and 1 U of Taq polymerase (Boehringer-Mannheim). Reactions were done in triplicate. Amplified products were separated on 3 % NuSeive agarose gels and the band intensity was compared to amplified actin bands. Samples processed in parallel but without reverse transcriptase added were used as negative controls.

28. GAL4-VP16 was fused in frame to the carboxyl termini of full-length Xenopus NRG-β1a, and was cloned by the PCR into pCDNA3.1/V5/His-TOPO (Invitrogen). Gal4-VP16 was fused to the C-terminal of NRG-β1a. Primer pairs used for amplification of Gal4-VP16 were 5': GTATA CCCAT ACCCG CCGAA GCTT and 3': CTTAT ACTCC ACCGT ACTCG TCAA; and for amplification of NRG-β1a: 5': ATGGC TGAGA AGAAG AAGGA AAAAG AA and 3': GTATG GGTAT ACAGC AATGG GGTCT TG. DNA sequences were confirmed by dideoxynucleotide sequencing.

29. pCAT (from G. Struhl) and p4luc (from R. Evans), which contain four Gal4 UAS elements fused to the bacterial CAT or firefly luciferase coding regions, respectively, were used as reporters. The following plasmids were co-transfected into 293T cells in various combinations: 10 mg p4luc (or pCAT), 20 mg of NRG-β1a-Gal4-VP16, 20 mg Gal4-VP16 and 2 mg of pCDNA-GFP (used to determine transfection efficiency). Total amounts of transfected DNA were kept constant by adding the appropriate amount of pCDNA. Lysates were prepared 48 hr

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after transfection for reporter expression assay. Where indicated, 0.5 mg/ml PMA, 0.5 mg/ml 4a-Phorbol 12,13-Didecanoate (4aPDD, biologically un-active phorbol), or 0.1% DMSO was added 8 hrs prior to lysis.

5

30. Epitope-tagged full-length or truncated forms of NRG- $\beta$ 1a or NRG- $\beta$ 1c-CD were prepared by the polymerase chain reaction (PCR), and cloned into pcDNA3.1/V5/His or pcDNA3.1/CT-GFP-TOPO (Invitrogen). The primer pair for fusing full-length CRD-NRG (a form) with the myc tag was: 5': ACCAT GTCTG AGGGA GCTGG CGGGA GGT and 3': ACTCA CCAGA TCTTC TTCAG AAATA AGTTT TTGTT CAGCA ATAGG GTCTT G. For fusing CRD-NRGa with the HA epitope we used 5': ACCAT GTCTG AGGGA GCTGG CGGGA GGT and 3': TCATA CAGCG TAGTC TGGGA CGTCG TATGG GTA. The PCR primer pair used to fuse full-length Ig-NRG (a form) with the GFP tag were: 5': AGCAT GGCTG AGAAG AAGAA GGAAA AA and 3': TACAG CAATG GGGTC TTGAT TCGTT ATTAC ACT. The PCR primer pair used to fuse the cytoplasmic domain containing NLS-1 (aa 295-390) to GFP was: 5': ATTAT GAAAA CCAAG AAACA GAGA and 3': GACCA TTACT CCAGC TGTGA CTTG. The PCR primer pair used to fuse the cytoplasmic domain lacking NLS-1 (aa 304-390) to GFP was 5': ATTAT GTTGA ATGAC CGTTT AAGA and 3': GACCA TTACT CCAGC TGTGA CTTG. DNA sequences were confirmed by dideoxynucleotide sequencing.

20

25

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31. Cytoplasmic, particulate and nuclear fractions from transfected cells were prepared using "Nuclear and Cytoplasmic extraction reagents" (PIERCE). 200 ml cytoplasmic fraction, 50 ml particulate fraction and 100 ml nuclear fraction were obtained from one 10 cm dish. Protein concentrations from each sample were measured a Bio-Rad protein assay kit based on the Bradford method



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(Bio-Rad Lab.). 40 mg nuclear, 40 ml particulate and 120 mg cytoplasmic protein were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes (Schleicher & Schuell), and probed with antibodies against NRG-1-CD, Histone or TIF5. Molecular mass was estimated by comparing the relative mobility of immunoreactive bands to prestained SDS-PAGE standards (Low Range, Bio-Rad).

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What is claimed is:

1. A cell comprising:
  - (1) a first recombinant nucleic acid comprising a first DNA region encoding a transmembrane isoform of a neuregulin protein linked in frame to a second DNA region encoding a transcription factor wherein expression of the first recombinant nucleic acid in the cell produces a transmembrane isoform of a neuregulin protein-transcription factor fusion protein, and
  - (2) a second recombinant nucleic acid comprising a promoter operatively linked to a reporter gene, wherein binding of the transcription factor portion of the fusion protein to the promoter activates expression of the reporter gene.
2. A method for detecting the presence of a protein in a biological sample, which protein has the following characteristics:
  - (1) selectively binds to a transmembrane isoform of a neuregulin protein and
  - (2) induces nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein, which comprises:
    - a) obtaining a biological sample from a subject;
    - b) providing a cell comprising:
      - (1) a first recombinant nucleic acid

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- comprising a first DNA region encoding a transmembrane isoform of a neuregulin protein linked in frame to a second DNA region encoding a transcription factor wherein expression of the first recombinant nucleic acid in the cell produces a transmembrane isoform of a neuregulin protein-transcription factor fusion protein, and
- (2) a second recombinant nucleic acid comprising a promoter operatively linked to a reporter gene, wherein binding of the transcription factor portion of the fusion protein to the promoter activates expression of the reporter gene;
- c) contacting the biological sample with the cell;
- d) measuring reporter gene expression level in the cell, wherein an increased reporter gene expression level compared to the reporter gene expression level measured in the cell in the absence of the biological sample is indicative of the presence of a protein in the biological sample, which protein has the following characteristics:
- (1) selectively binds to a transmembrane isoform of a neuregulin protein and
- (2) induces nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein.

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3. A method for determining the amount of a protein in a biological sample, which protein has the following characteristics:
- 5 (1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) induces nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein, which comprises:
- 10 a) obtaining a biological sample from a subject;
- b) providing a cell comprising:
- 15 (1) a first recombinant nucleic acid comprising a first DNA region encoding a transmembrane isoform of a neuregulin protein linked in frame to a second DNA region encoding a transcription factor wherein expression of the first recombinant
- 20 nucleic acid in the cell produces a transmembrane isoform of a neuregulin protein-transcription factor fusion protein, and
- 25 (2) a second recombinant nucleic acid comprising a promoter operatively linked to a reporter gene, wherein binding of the transcription factor portion of the fusion protein to the promoter activates expression of the
- 30 reporter gene;
- c) contacting the biological sample with the cell;
- d) measuring reporter gene expression

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level in the cell;

- e) comparing the reporter gene expression level measured in step d) with a reporter gene expression level measured in multiple samples and multiple different known amounts of protein which selectively binds to a transmembrane isoform of a neuregulin protein, thereby determining the amount of a protein in a biological sample, which protein has the following characteristics:
- (1) selectively binds to a transmembrane isoform of a neuregulin protein and
  - (2) induces nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein.

4. A method for early detection of cancer in a subject which comprises:

- a) obtaining a biological sample from a first subject;
- b) providing a cell comprising:
  - (1) a first recombinant nucleic acid comprising a first DNA region encoding a transmembrane isoform of a neuregulin protein linked in frame to a second DNA region encoding a transcription factor wherein expression of the first recombinant nucleic acid in the cell produces a transmembrane isoform of a neuregulin protein-transcription factor fusion protein; and

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- 5 (2) a second recombinant nucleic acid comprising a promoter operatively linked to a reporter gene, wherein binding of the transcription factor portion of the fusion protein to the promoter activates expression of the reporter gene;
- 10 c) contacting the biological sample with the cell;
- d) measuring reporter gene expression level in the cell;
- 15 e) comparing the reporter gene expression level in d) with a reporter gene expression level measured in a biological sample which is from a second subject without cancer, wherein a higher reporter gene expression level in the biological sample from the first subject is indicative of the first subject having cancer.
- 20
5. A method for identifying a compound, which compound has the following characteristics:
- 25 (1) selectively binds to a transmembrane isoform of a neuregulin protein and
- (2) induces nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein, which
- 30 comprises:
- a) admixing a compound with a cell comprising:
- (1) a first recombinant nucleic acid

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5 comprising a first DNA region  
encoding a transmembrane isoform of  
a neuregulin protein linked in frame  
to a second DNA region encoding a  
transcription factor wherein  
expression of the first recombinant  
nucleic acid in the cell produces a  
transmembrane isoform of a neuregulin  
protein-transcription factor fusion  
10 protein, and

(2) a second recombinant nucleic acid  
comprising a promoter operatively  
linked to a reporter gene, wherein  
binding of the transcription factor  
portion of the fusion protein to the  
15 promoter activates expression of the  
reporter gene;

b) measuring reporter gene expression level  
in the cell, wherein an increased reporter  
gene expression level compared to the  
reporter gene expression level in the cell  
in the absence of the compound is  
indicative of a compound having the  
following characteristics: (1) selectively  
25 binds to a transmembrane isoform of a  
neuregulin protein and (2) induces nuclear  
translocation of a cytoplasmic domain of  
the transmembrane isoform of the  
neuregulin protein.

30

6. A method for identifying a compound, which  
compound has the following  
characteristics: (1) selectively binds to

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a transmembrane isoform of a neuregulin protein in a cell and (2) inhibits nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein in a cell, which comprises:

- a) admixing a compound with a cell transfected with (1) a recombinant nucleic acid comprising a first DNA sequence encoding a transmembrane isoform of a neuregulin protein linked in frame to a second DNA sequence encoding a transcription factor wherein expression of the recombinant nucleic acid produces a fusion protein, and (2) a recombinant nucleic acid comprising a promoter region operatively linked to a reporter gene, wherein the transcription factor portion of the fusion protein binds to the promoter region thereby activating expression of the reporter gene;
- b) measuring reporter gene expression level in the cell, wherein a decreased reporter gene expression level compared to the reporter gene expression level in the cell in the absence of the compound is indicative of the presence of a compound, which compound has the following characteristics: (1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) inhibits nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the



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neuregulin protein.

7. A pharmaceutical composition comprising:
- 5 i) a compound which (1) selectively binds to a transmembrane isoform of a neuregulin protein and (2) inhibits nuclear translocation of a cytoplasmic domain of the transmembrane isoform of the neuregulin protein, determined to do so by
- 10 the method of claim 6; and
- ii) a carrier.
8. A method for treating cancer in a subject which comprises administering to the
- 15 subject a therapeutically effective amount of a compound identified by the method of claim 6 or the pharmaceutical composition of claim 7 so as to treat cancer in a subject.
- 20
9. The cell of claim 1, wherein the reporter gene encodes a green fluorescent protein, a  $\beta$ -galactosidase, a luciferase, a chloramphenicol acetyltransferase, a  $\beta$
- 25 glucuronidase, a neomycin phosphotransferase, or a guanine xanthine phosphoribosyltransferase.
10. The method of claim 2, wherein the reporter
- 30 gene encodes a green fluorescent protein, a  $\beta$ -galactosidase, a luciferase, a chloramphenicol acetyltransferase, a  $\beta$  glucuronidase, a neomycin phosphotransferase, or a guanine

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xanthine phosphoribosyltransferase.

11. The method of claim 2, 3, or 4, wherein the  
protein is ErbB1/HER1 ErbB2/HER2, ErbB3/HER3,  
5 or ErbB4/HER4.
12. The cell of claim 1, wherein the transmembrane  
isoform of the neuregulin is a cysteine rich  
domain neuregulin (CRD-NRG).  
10
13. The method of claim 2, 3, 4, 5, 6, or 7,  
wherein the transmembrane isoform of the  
neuregulin is a cysteine rich domain neuregulin  
(CRD-NRG).  
15
14. The method of claim 2, 3, 4, 5, 6, or 7,  
wherein the cytoplasmic domain is a cyt-a, cyt-  
b, or cyt-c domain.
- 20 15. The cell of claim 1, wherein the promoter  
region is a gal4 upstream activator sequence.
16. The method of claim 2, 3, 4, 5, or 6, wherein  
the promoter region is a gal4 upstream  
25 activator sequence.
17. The cell of claim 1, wherein the transcription  
factor is a gal4/VP16 transcription factor.
- 30 18. The method of claim 2, 3, 4, 5, or 6, wherein  
the transcription factor is a gal4/VP16  
transcription factor.

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19. The method of claim 2, 3, 4, 5, or 6, wherein the cell is a human embryonic kidney cell.
20. The method of claim 4 or 8, wherein the cancer is a breast cancer, an ovarian cancer, a prostate cancer, a glioma, or a neuroblastoma.
21. A cell which comprises (1) a first recombinant nucleic acid comprising a first DNA region encoding a ligand binding domain of a protein linked in frame to a second DNA sequence encoding a channel forming domain a  $\alpha$ -7 type neuronal nicotine receptor linked in frame to a third DNA region encoding a transcription factor wherein expression of the first recombinant nucleic acid produces a ligand binding domain of a protein-channel forming domain a  $\alpha$ -7 type neuronal nicotine receptor-transcription factor fusion protein and (2) a second recombinant nucleic acid comprising a promoter operatively linked to a reporter gene, wherein the transcription factor binds to the promoter thereby activating expression of the reporter gene.
22. A method for detecting the presence of a molecule in a biological sample, which molecule selectively binds to a ligand gated ion channel receptor, which comprises:
- obtaining a biological sample from a subject;
  - contacting the biological sample with a cell which comprises (1) a first

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5 recombinant nucleic acid comprising a  
first DNA region encoding a ligand binding  
domain of a protein linked in frame to a  
second DNA sequence encoding a channel  
forming domain a  $\alpha$ -7 type neuronal  
nicotine receptor linked in frame to a  
third DNA region encoding a transcription  
factor wherein expression of the first  
recombinant nucleic acid produces a ligand  
10 binding domain of a protein-channel  
forming domain a  $\alpha$ -7 type neuronal  
nicotine receptor-transcription factor  
fusion protein and (2) a second  
recombinant nucleic acid comprising a  
15 promoter operatively linked to a reporter  
gene, wherein the transcription factor  
binds to the promoter thereby activating  
expression of the reporter gene;

20 c) measuring reporter gene expression level  
in the cell, wherein an increased reporter  
gene expression level compared to the  
reporter gene expression level measured in  
the cell in the absence of the biological  
sample is indicative of the presence of  
25 a molecule which selectively binds to a  
ligand gated ion channel receptor in a  
biological sample.

23. A method for determining the amount of a  
30 molecule in a biological sample, which molecule  
selectively binds to a ligand gated ion channel  
receptor, which comprises:

a) obtaining a biological sample from a

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subject;

- b) providing a cell which comprises (1) a first recombinant nucleic acid comprising a first DNA region encoding a ligand binding domain of a protein linked in frame to a second DNA sequence encoding a channel forming domain a  $\alpha$ -7 type neuronal nicotine receptor linked in frame to a third DNA region encoding a transcription factor wherein expression of the first recombinant nucleic acid produces a ligand binding domain of a protein-channel forming domain a  $\alpha$ -7 type neuronal nicotine receptor-transcription factor fusion protein and (2) a second recombinant nucleic acid comprising a promoter operatively linked to a reporter gene, wherein the transcription factor binds to the promoter thereby activating expression of the reporter gene;
- c) contacting the biological sample with the cell;
- d) measuring reporter gene expression level;
- e) comparing the reporter gene expression level measured in step d) with a reporter gene expression level measured in multiple samples and multiple different known amounts of molecule which selectively binds to a ligand gated ion channel receptor, thereby determining the amount of a molecule, which molecule selectively binds to a ligand gated ion channel receptor.

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24. A method for early detection of a neurodegenerative disease in a subject which comprises:

- 5 a) obtaining a biological sample from a first subject;
- 10 b) contacting the sample with a cell which comprises (1) a first recombinant nucleic acid comprising a first DNA region encoding a ligand binding domain of a protein linked in frame to a second DNA sequence encoding a channel forming domain a  $\alpha$ -7 type neuronal nicotine receptor linked in frame to a third DNA region encoding a transcription factor wherein
- 15 expression of the first recombinant nucleic acid produces a ligand binding domain of a protein-channel forming domain a  $\alpha$ -7 type neuronal nicotine receptor-transcription factor fusion protein and
- 20 (2) a second recombinant nucleic acid comprising a promoter operatively linked to a reporter gene, wherein the transcription factor binds to the promoter thereby activating expression of the
- 25 reporter gene;
- c) measuring reporter gene expression level in the cell;
- 30 d) comparing the reporter gene expression level in c) with a reporter gene expression level in a sample which is from a second subject without neurodegenerative disease, a lower amount in the sample from the first subject being indicative of the

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first subject having a neurodegenerative disease.

25. The cell of claim 21, wherein the ligand  
5 binding domain specifically binds to a  
neuregulin receptor, neurotransmitter, or  
neurotransmitter metabolite.
26. The method of claim 22, 23, or 24, wherein the  
10 ligand binding domain specifically binds to a  
neuregulin receptor, neurotransmitter, or  
neurotransmitter metabolite.
27. The cell of claim 21, wherein the channel  
15 forming domain is a calcium channel forming  
domain of a  $\alpha$ -7 type neuronal nicotine  
receptor.
28. The method of claim 22, 23, or 24, wherein the  
20 channel forming domain is a calcium channel  
forming domain of a  $\alpha$ -7 type neuronal nicotine  
receptor.
29. The method of claim 2, 3, 4, 22, 23, or 24,  
25 wherein the biological sample is blood,  
cerebrospinal fluid (CSF), plasma, sputum,  
amniotic fluid, ascites fluid, breast aspirate,  
saliva, urine, lung lavage, or cell lysate or  
extract derived from a biopsy.
30. The method of claim 22, 23, or 24, wherein the  
30 cell is a human embryonic kidney cell.

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31. The cell of claim 21, wherein the promoter region is a cAMP responsive element (CRE) binding site.
- 5 32. The method of claim 22, 23, or 24, wherein the promoter region is a cAMP responsive element (CRE) binding site.
- 10 33. The cell of claim 21, wherein the transcription factor is a CREB transcription factor.
34. The method of claim 22, 23, or 24, wherein the transcription factor is a CREB transcription factor.
- 15 35. The method of claim 24 wherein the neurodegenerative disease is Alzheimer's disease or Parkinson's Disease.
- 20 36. The method of claim 24 or 42, wherein the neurodegenerative disease is associated with aging, amyotrophic lateral sclerosis, dentatorubral and pallidolysian atrophy, Huntington's disease, Machado-Joseph disease, multiple sclerosis, muscular dystrophy, senility, spinocerebellar ataxia type I, spinobulbar muscular atrophy, stroke, trauma.
- 25 37. The cell of claim 1 or claim 21, wherein the cell is a human embryonic kidney cell.
- 30 38. The cell of claim 1 or 21, wherein the cell is



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a bacterial cell, a yeast cell, a fungal cell, an insect cell, a nematode cell, a plant or animal cell.

- 5        39.        The method of claim 2, 3, 4, 5, 6, 22, 23, or 24, wherein the cell is a bacterial cell, a yeast cell, a fungal cell, an insect cell, a nematode cell, a plant or animal cell.
- 10       40.        The pharmaceutical composition of claim 7 or the method of claim 42, wherein the carrier comprises saline, sodium acetate, ammonium acetate, a virus, a liposome, a microencapsule, a polymer encapsulated cell, a retroviral
- 15       vector, a diluent, or an isotonic, pharmaceutically acceptable buffer solution.
- 20       41.        The method of claim 5 or 6, wherein the compound is a peptide, a peptidomimetic, a nucleic acid, an organic molecule, an inorganic chemical, or a lipid-based compound.
- 25       42.        A method for treating a neurodegenerative disease in a subject which comprises administering to the subject a therapeutically effective amount of the compound of claim 5 and a carrier so as to treat a neurodegenerative disease in a subject.
- 30       43.        The method of claim 2, 3, 4, 8, 22, 23, 24, or 42, wherein the subject is a mammal.

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44. The method of claim 42, wherein the mammal is a human.

5 45. The cell of claim 21, wherein the molecule is a neuregulin receptor, a neurotransmitter, or a neurotransmitter metabolite.

10 46. The method of claim 22, 23, or 24, wherein the molecule is a neuregulin receptor, a neurotransmitter, or a neurotransmitter metabolite.

15 47. The method of claim 8 or 42, wherein the administering is via intralesional, intraperitoneal, intramuscular or intravenous injection; infusion; liposome-mediated delivery; topical, nasal, oral, anal, ocular or otic delivery.

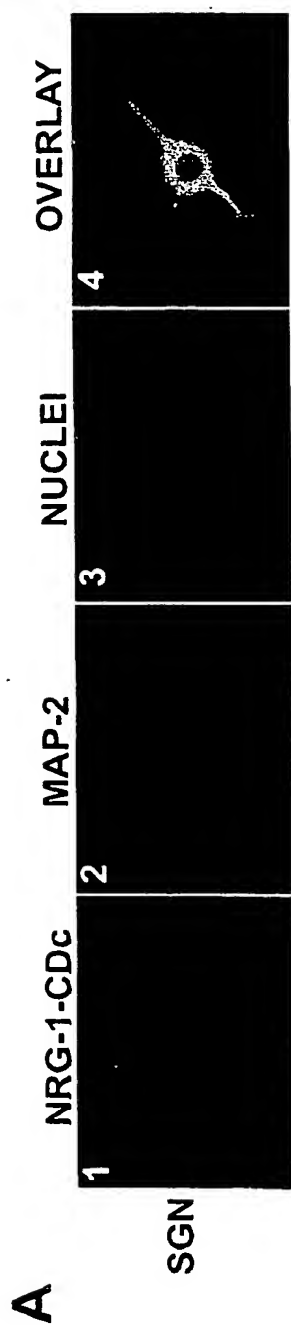
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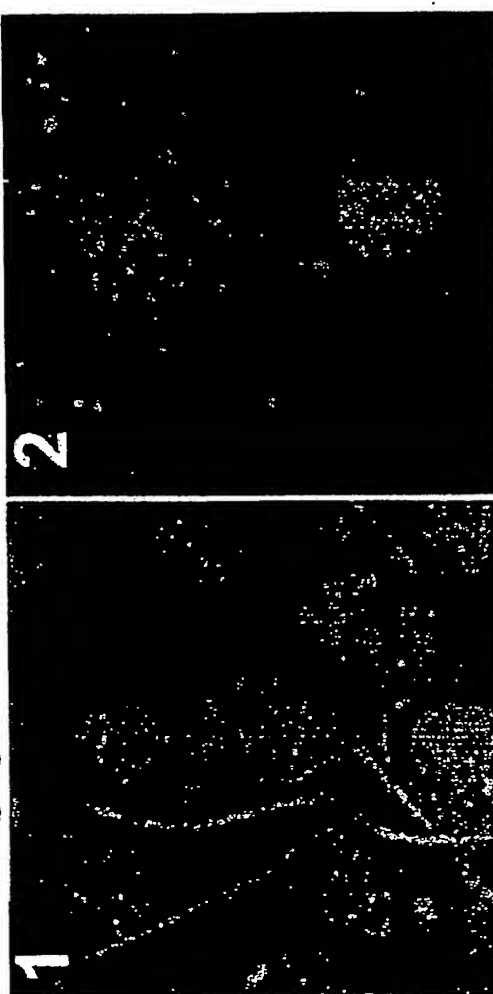
1/35

**FIGURE 1-1 A**

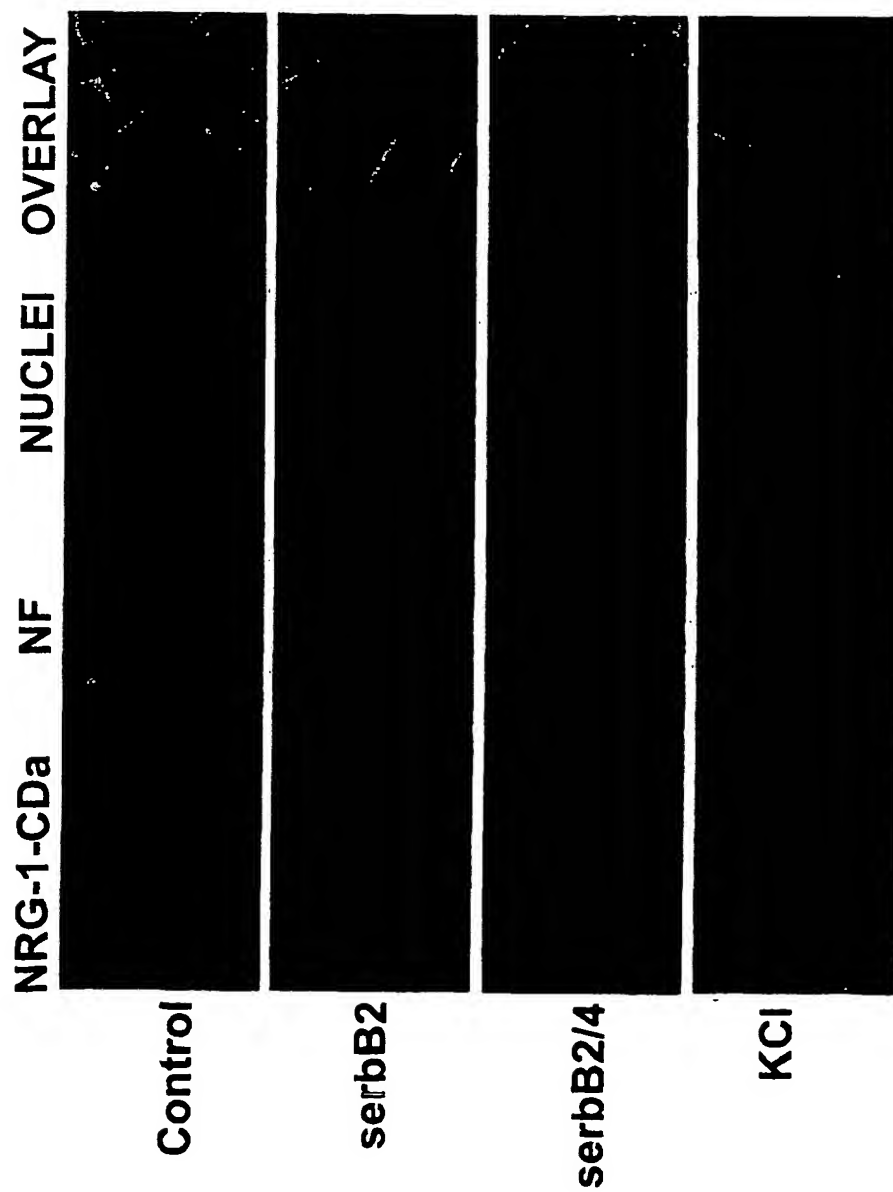


**FIGURE 1-1 B**

**SGN HIPPOCAMPAL**

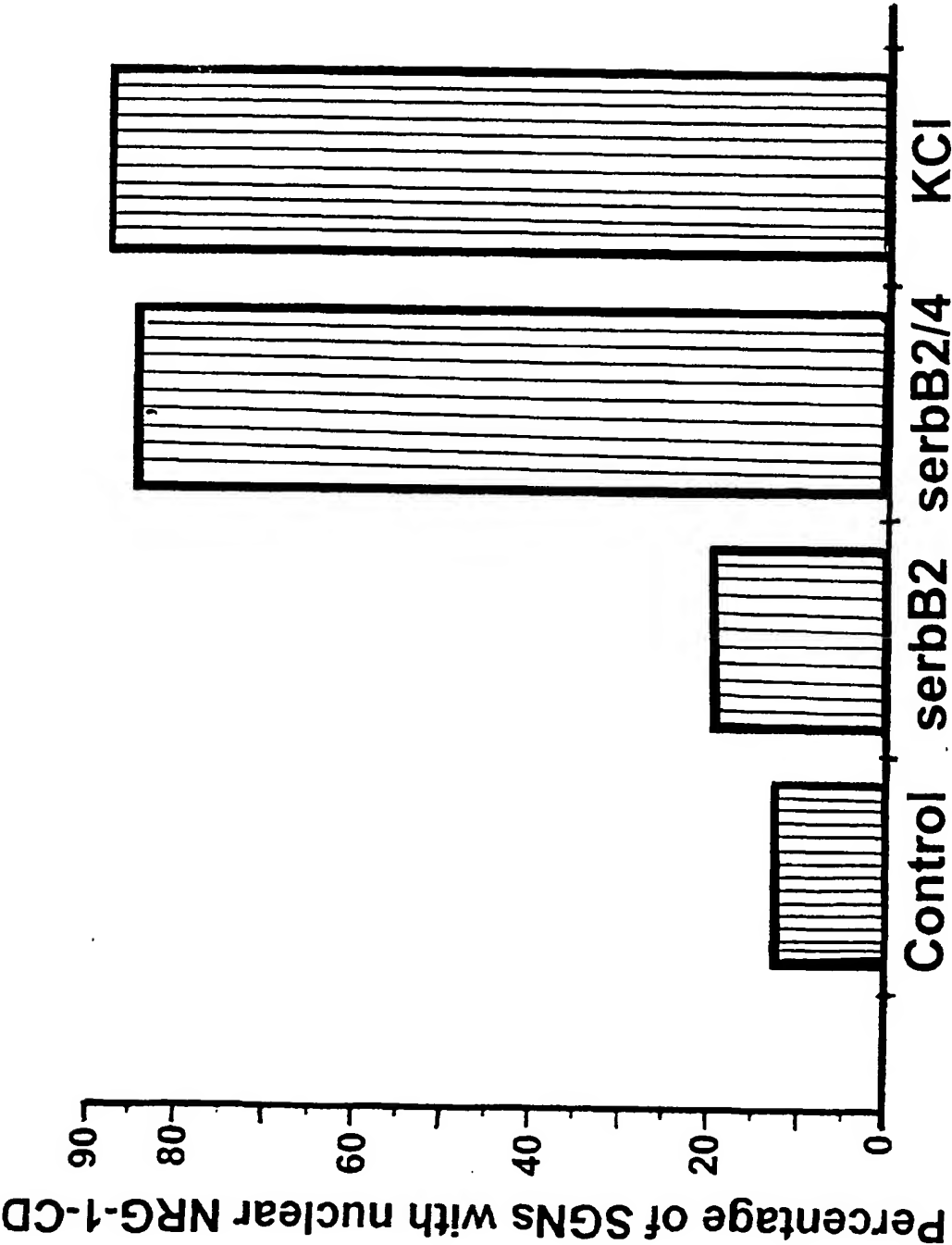


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**FIGURE 1-2 C**

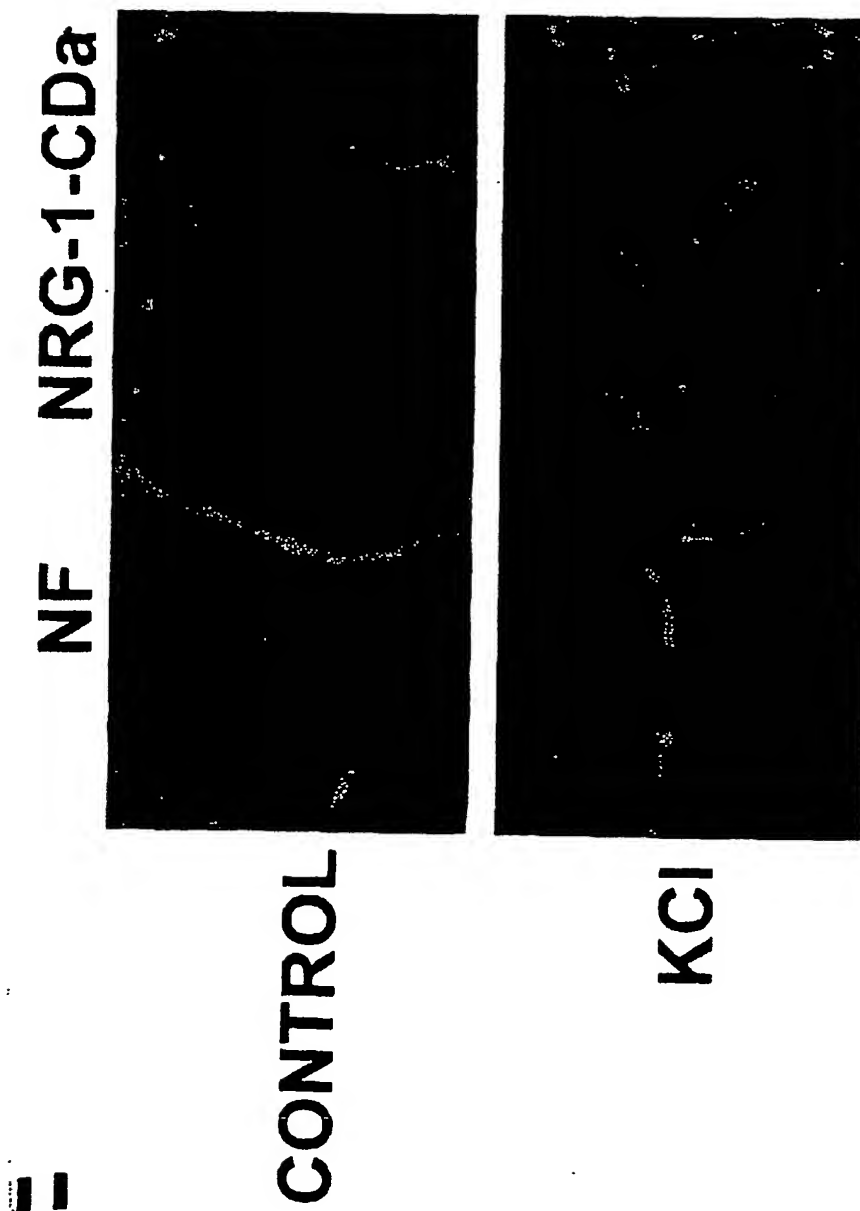
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FIGURE 1-2 D



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FIGURE 1-3 E



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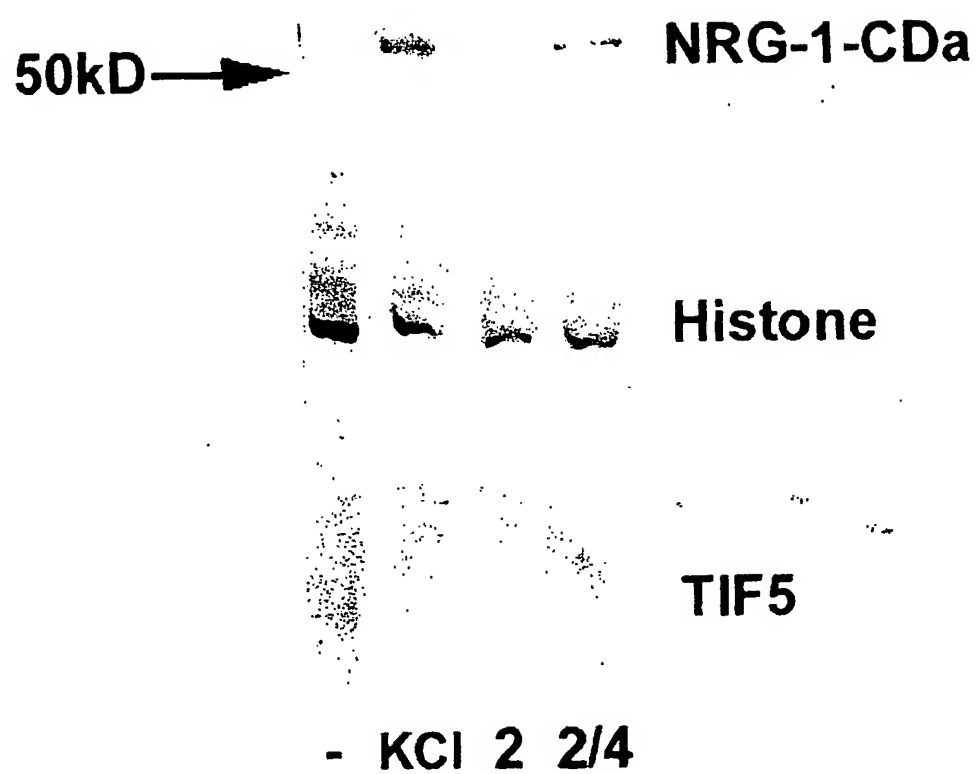
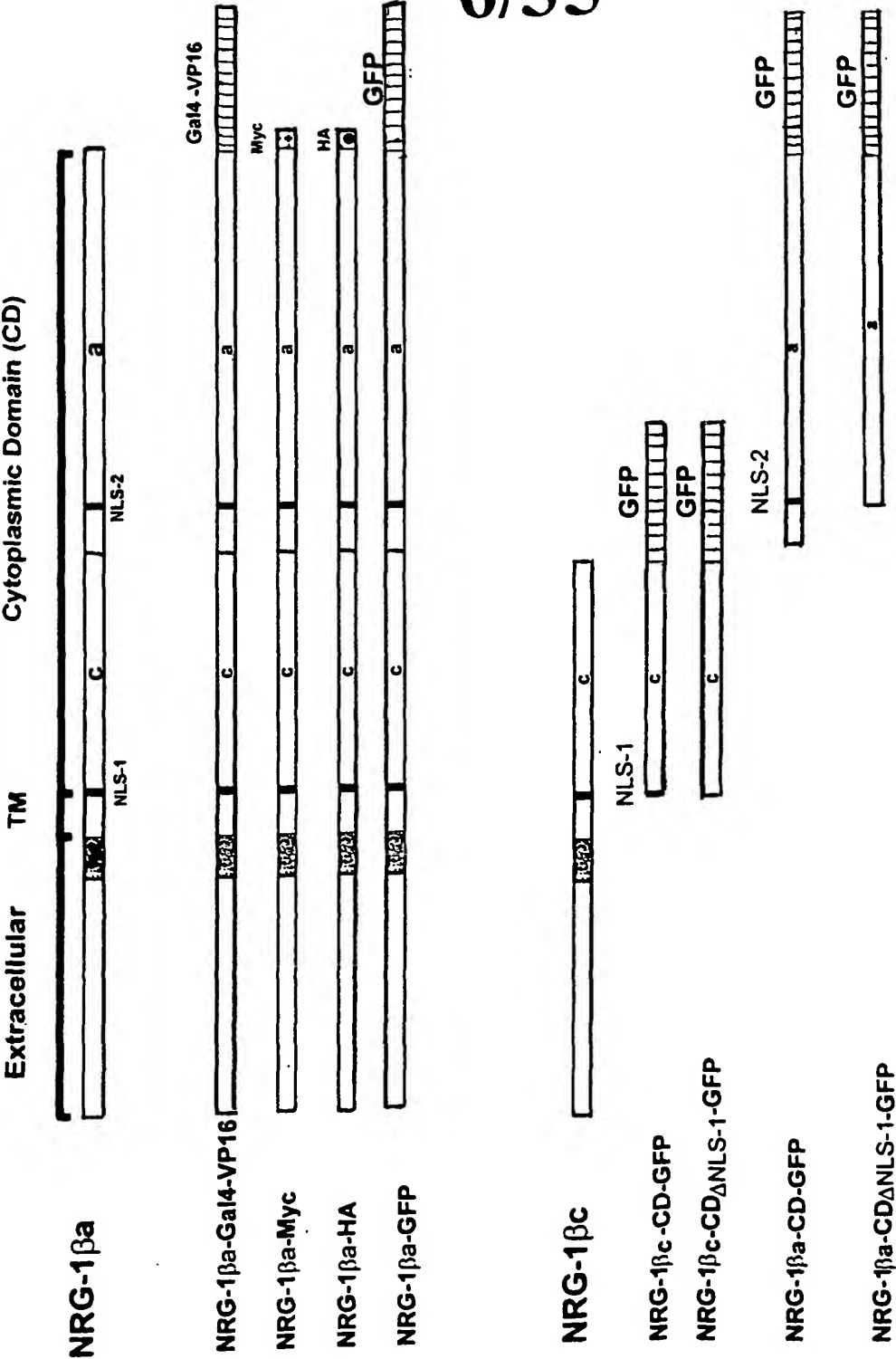
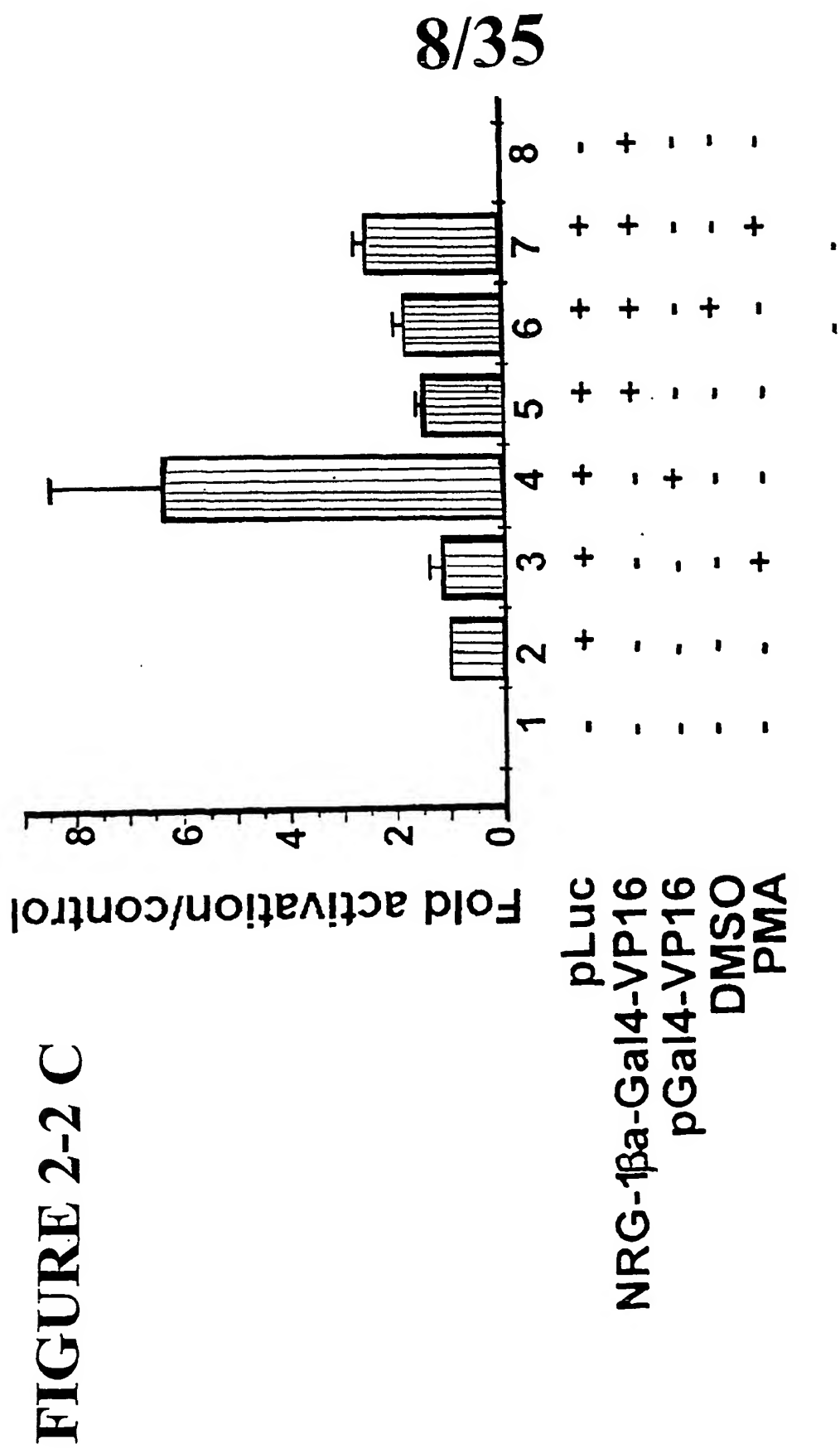
**FIGURE 1-3 F**

FIGURE 2-1 A

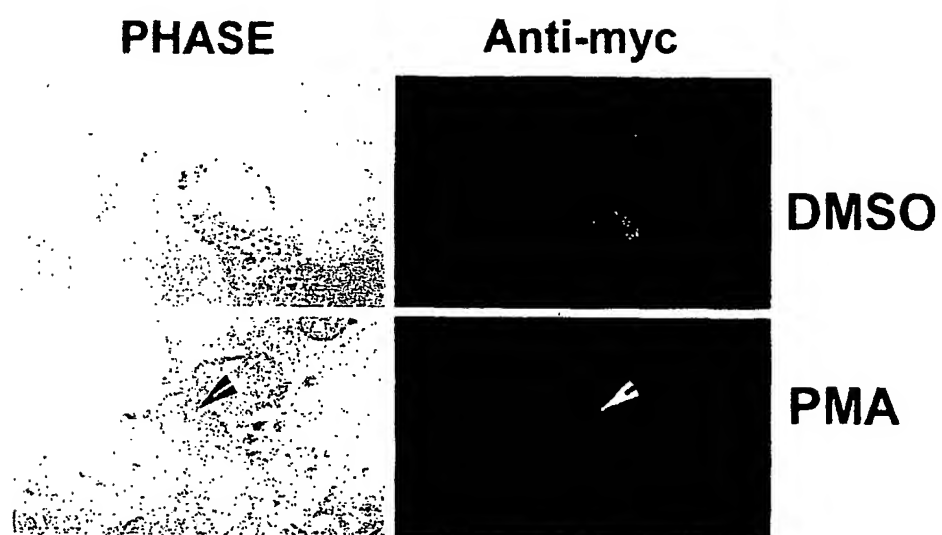






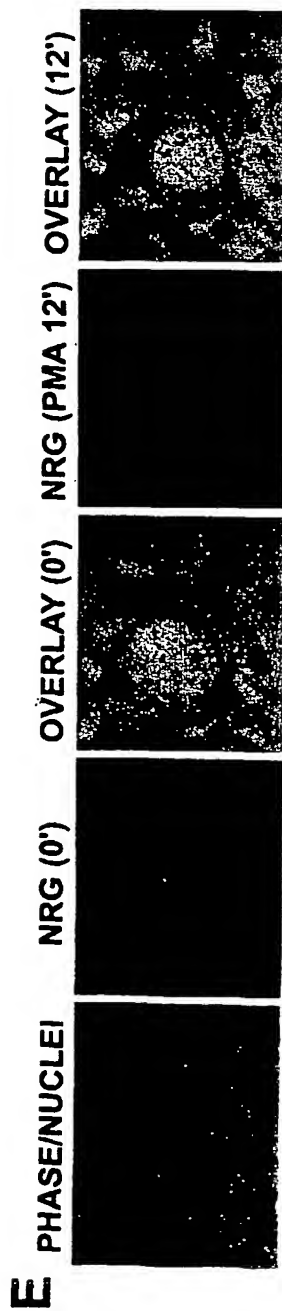


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**FIGURE 2-2 D**

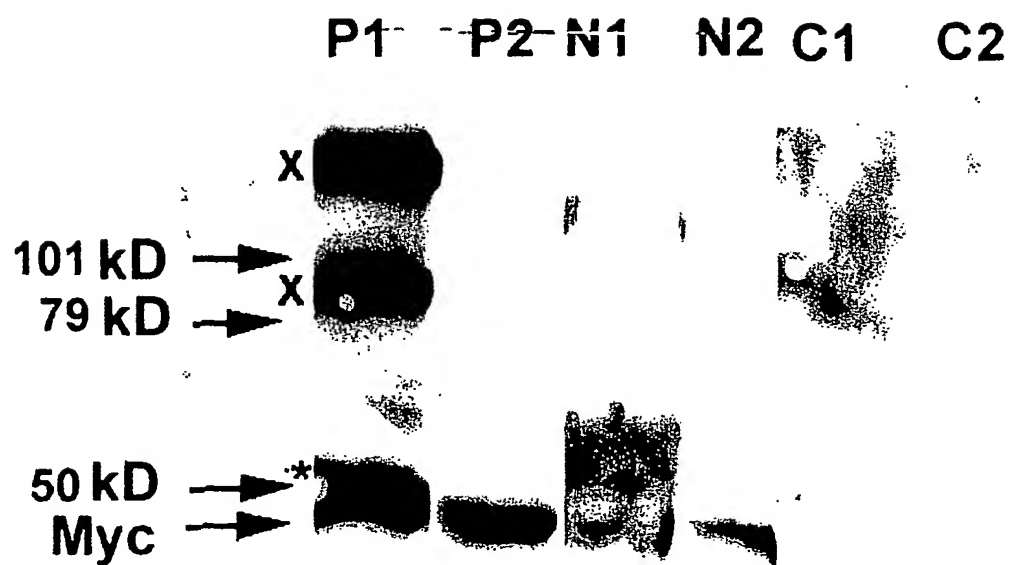
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# FIGURE 2-3 E



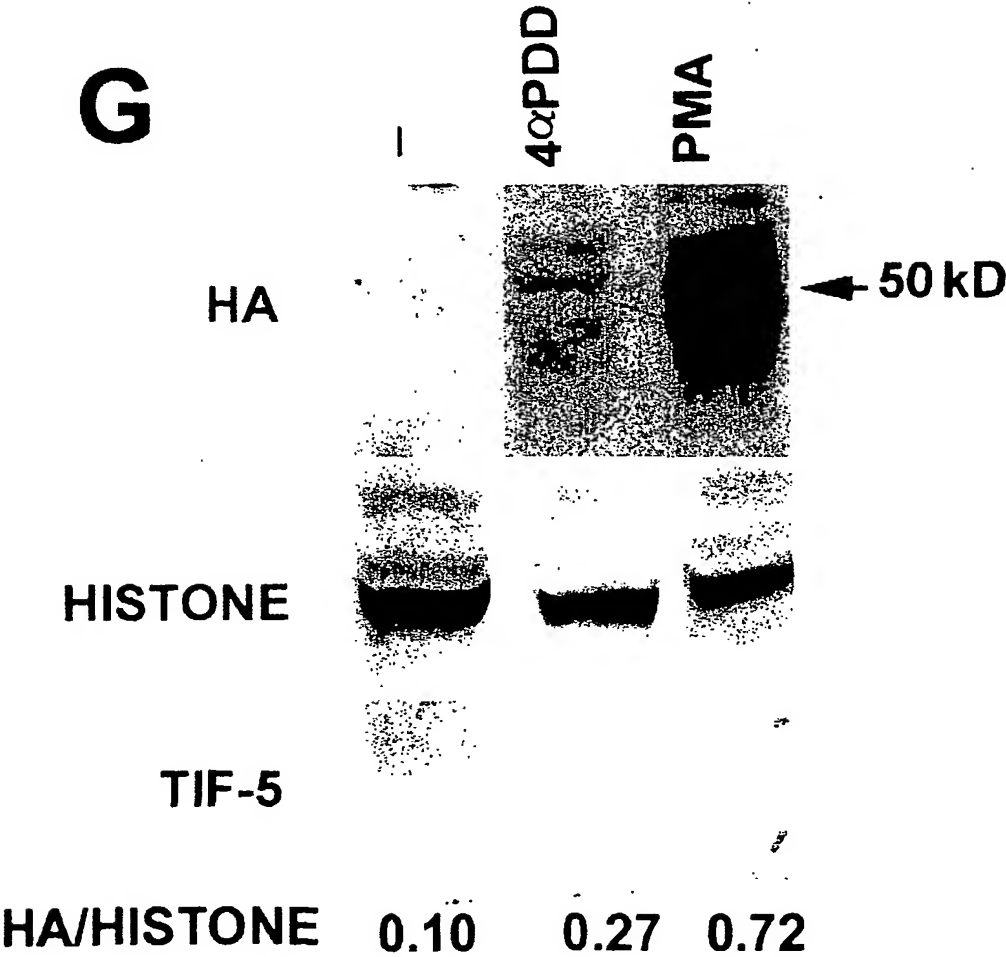
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FIGURE 2-3 F

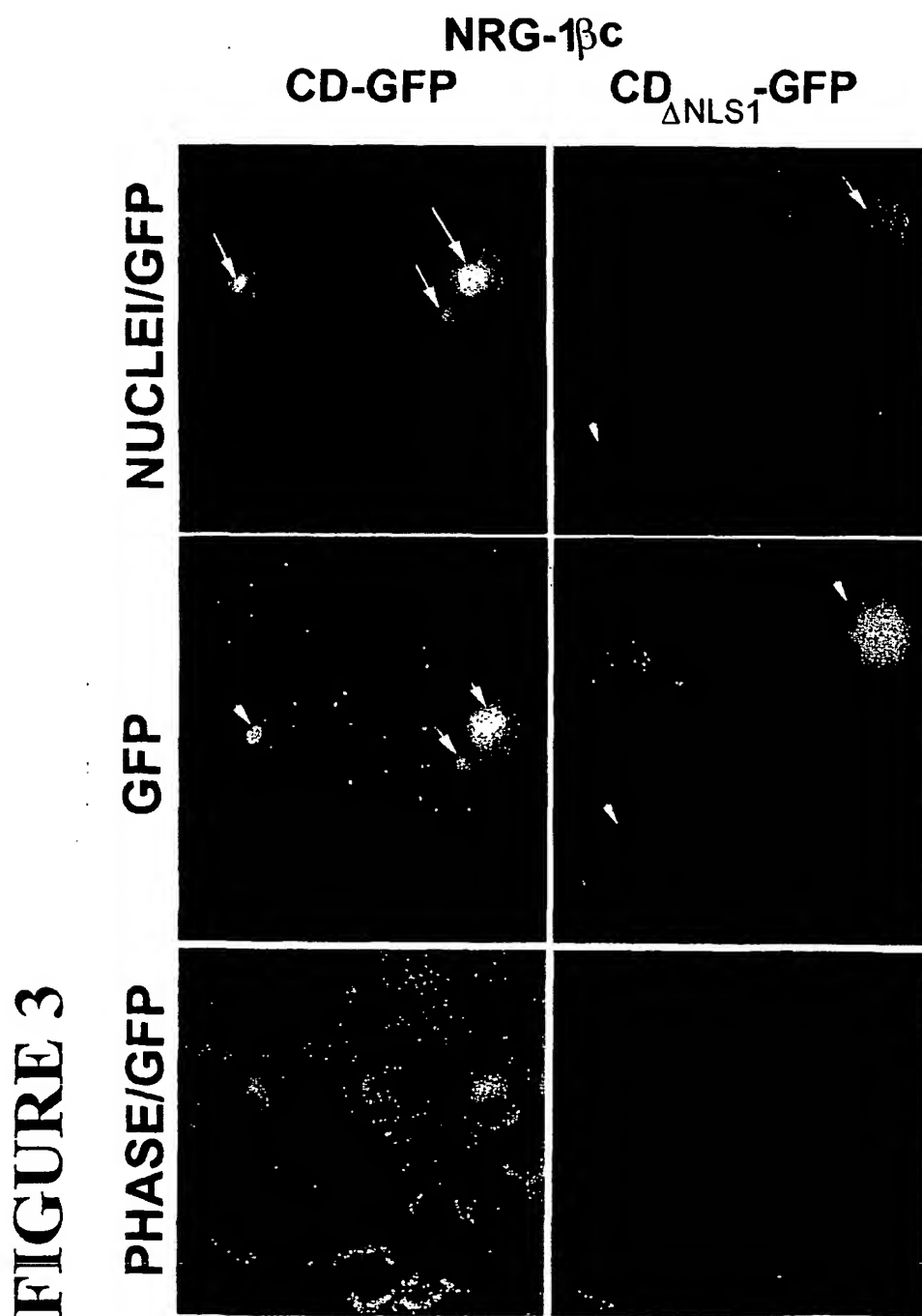


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FIGURE 2-3 G

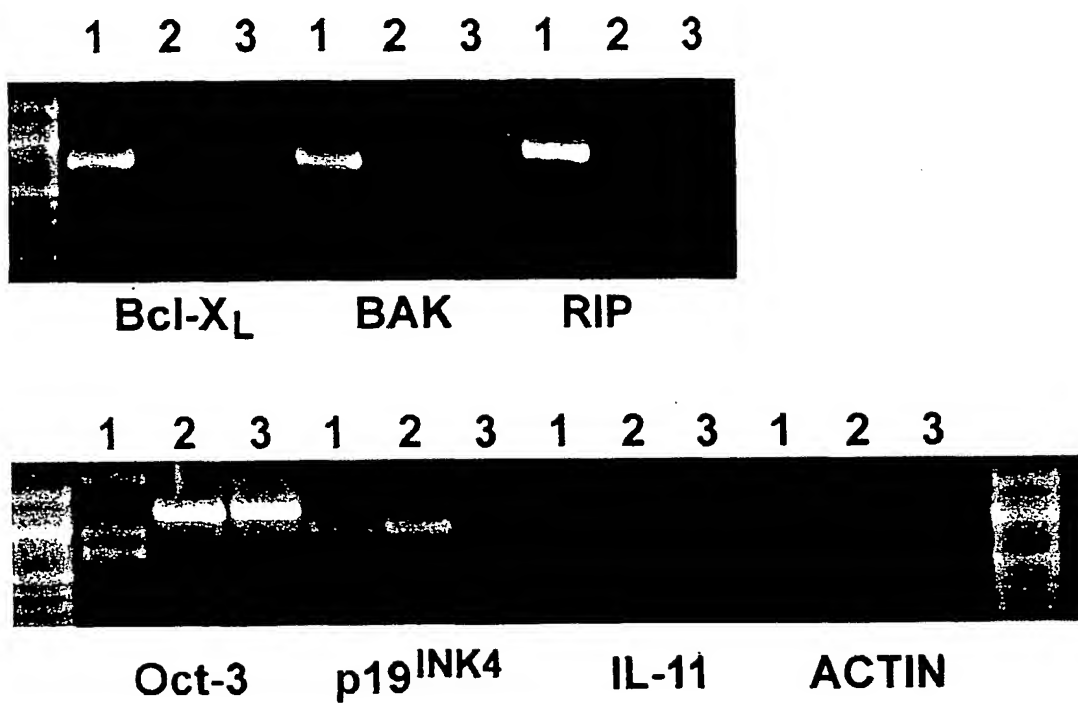


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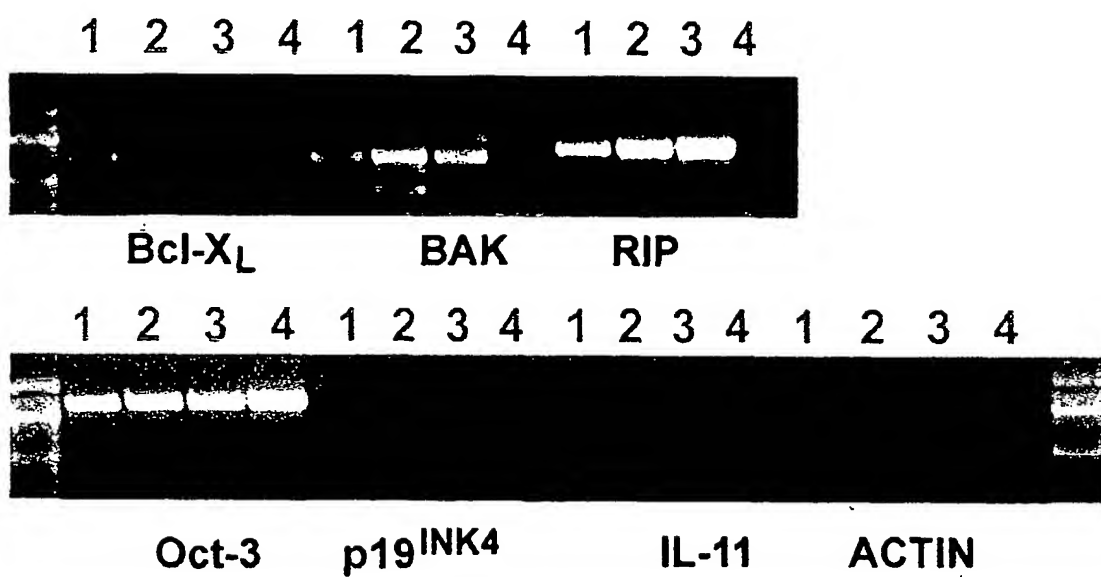
14/35

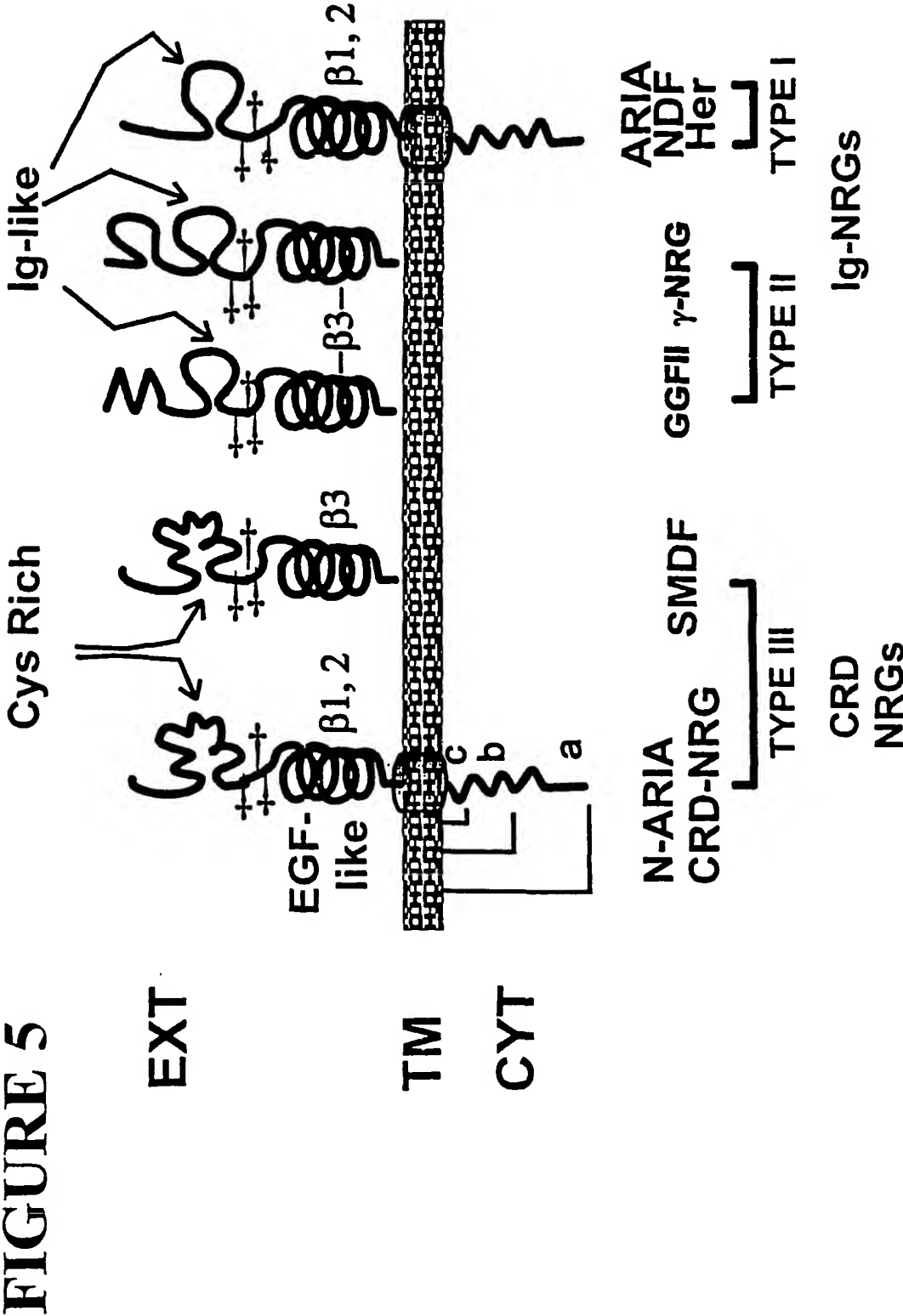
## FIGURE 4A



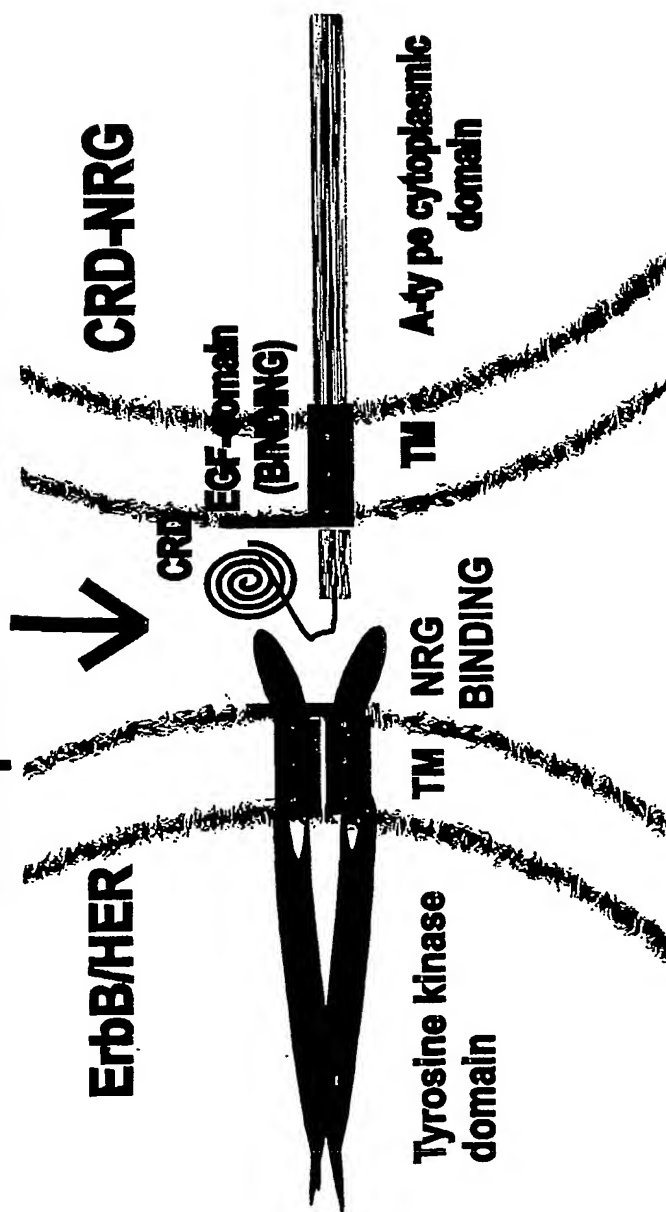


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**FIGURE 4B**



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**FIGURE 6****Extracellular site of ligand-receptor interactions**

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## FIGURE 7

## 1. CRUDE SAMPLE + DETECTOR CELLS



1: NRG CYT-A "BACK-SIGNALING" IS USED as a DETECTOR of CIRCULATING ERBs (↑ IN SPECIFIC CA'S, NEURODEGENERATION)

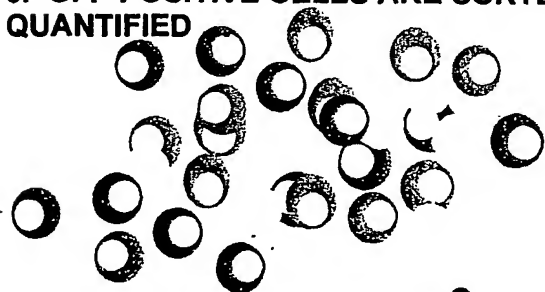


2: THE HIGH CALCIUM PERMEABILITY OF  $\alpha 7$  nAChRs IS USED as a DETECTOR of CIRCULATING NEUROTROPHIC FACTORS OR TRANSMITTERS

2. DETECTION OF THE PRIMARY SIGNAL IS RAPID & QUANTIFIABLE. AN AMPLIFIED "READ OUT" IS PROVIDED BY USE OF CHIMERIC GENE CONSTRUCTS LINKED TO GFP.

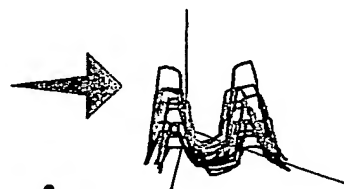


## 3. GFP POSITIVE CELLS ARE SORTED AND FLUORESCENCE IS QUANTIFIED



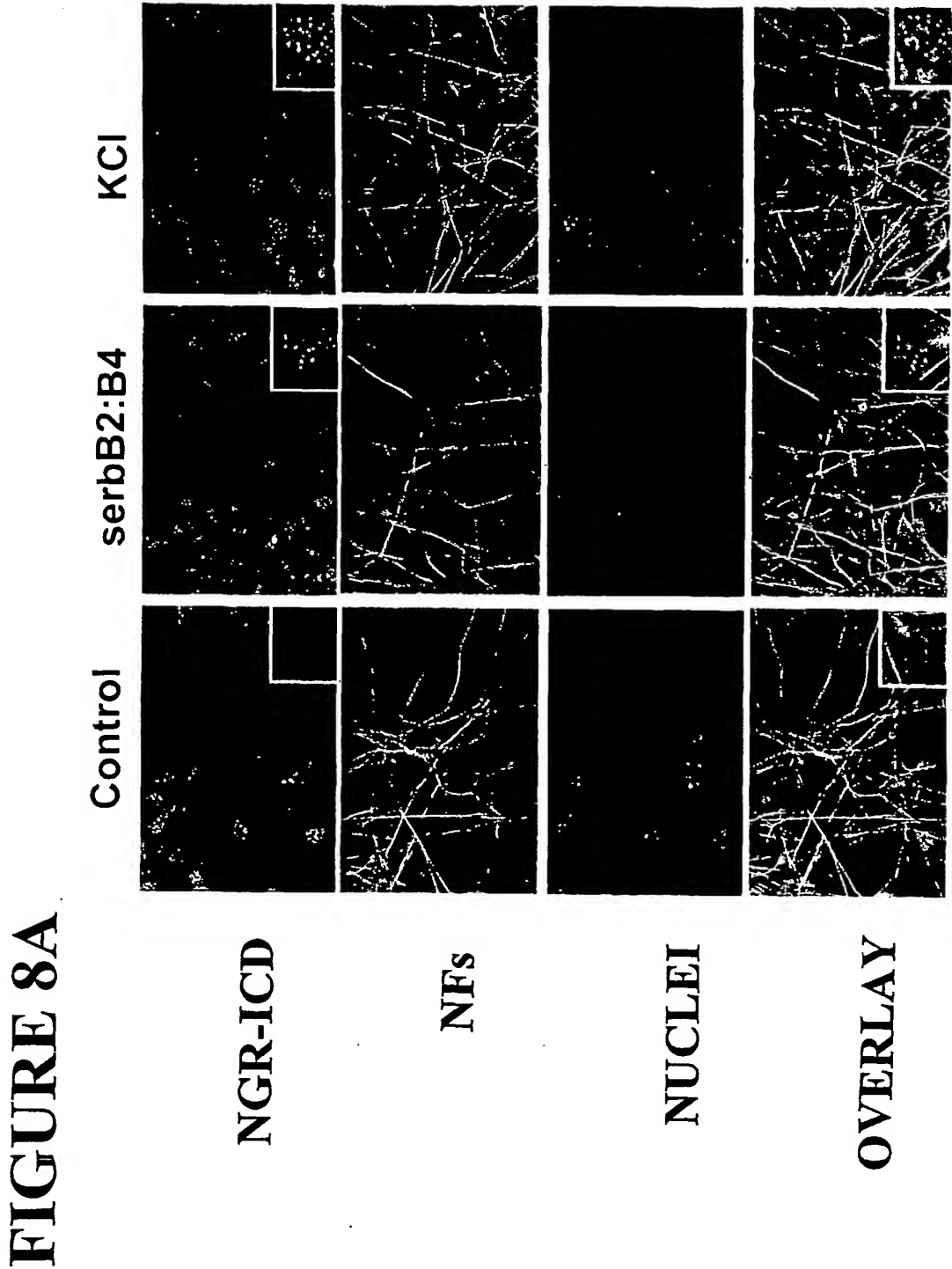
NRG-CYT a CHIMERA WITH VP16 TRANSACTIVATION DOMAIN.

NRG BINDING DOMAIN



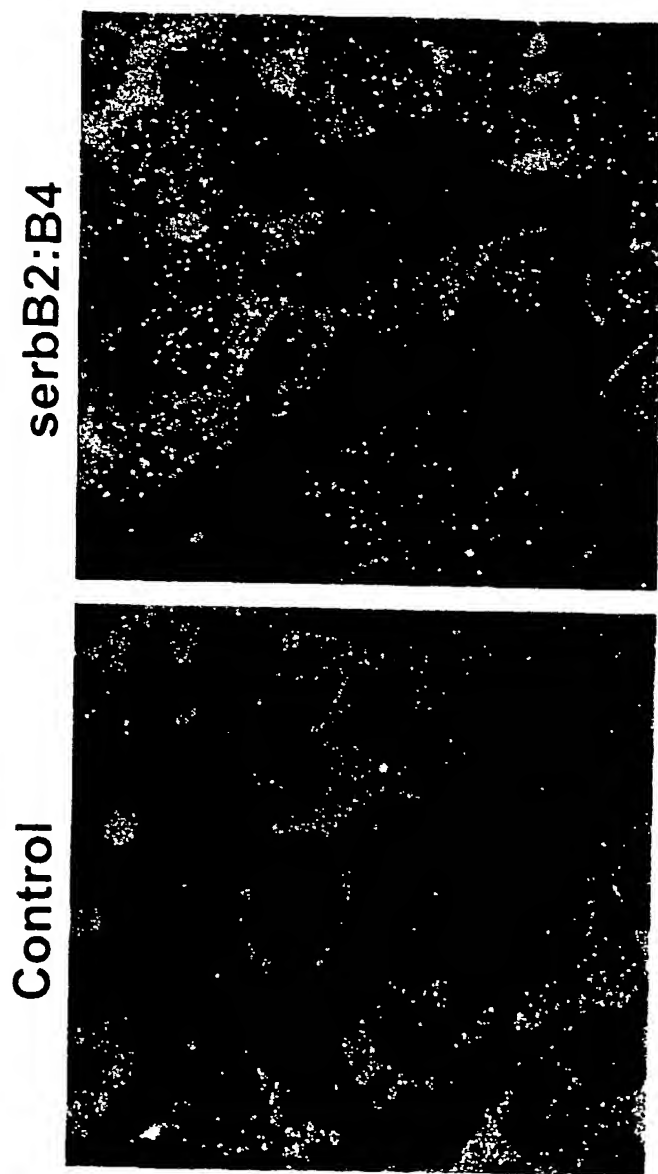
CHIMERA OF A SPECIFIC BINDING DOMAIN SEQUENCE WITH THE CHANNEL FORMING PORTION OF  $\alpha 7$  nAChR – (eg: The first 224 AA'S of the  $\alpha 7$  nAChR subunit sequence is replaced with the N terminal)

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**FIGURE 8B**



**OVERLAY :  
NGR-ICD  
NUCLEI**

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FIGURE 8C

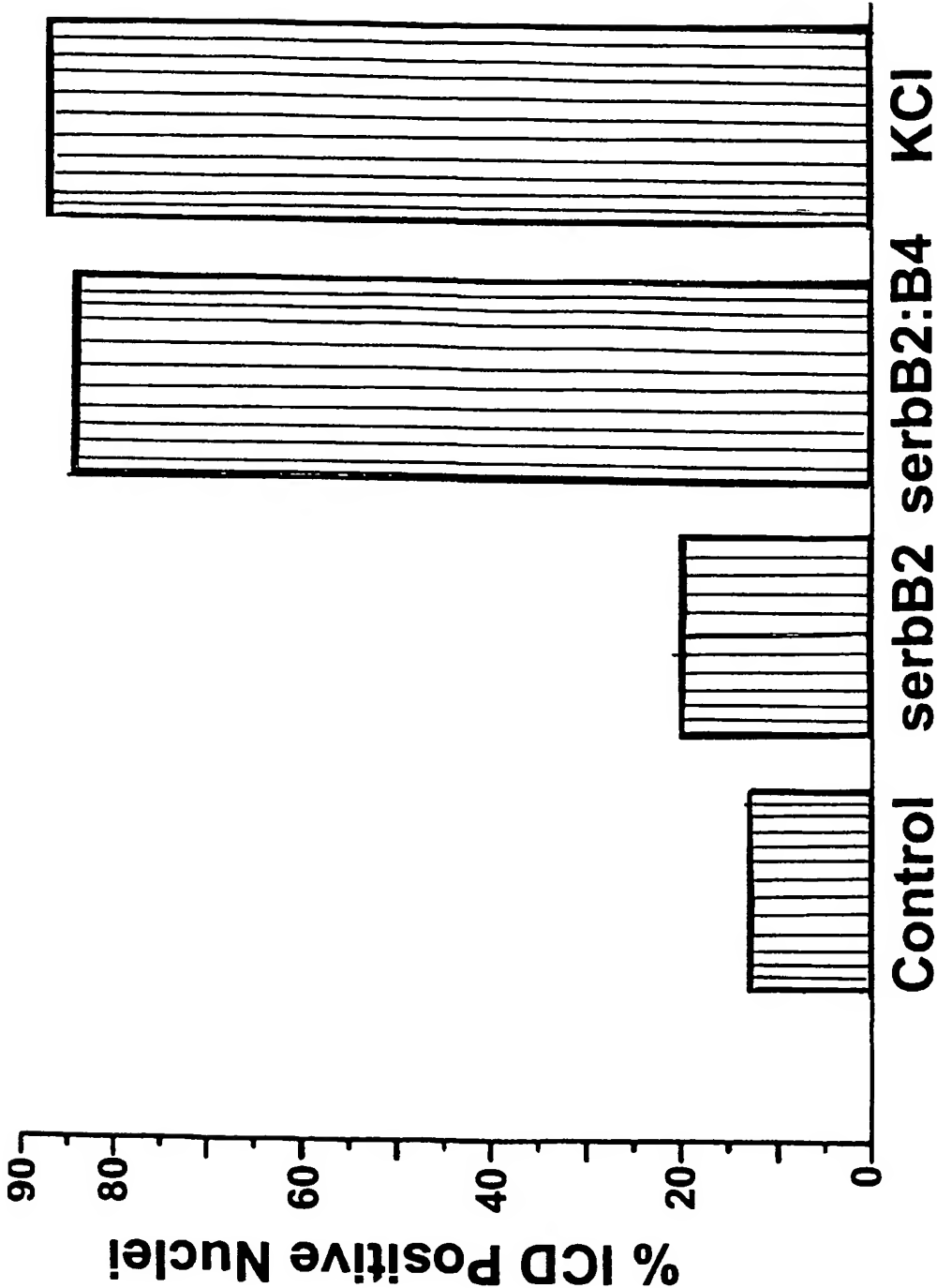
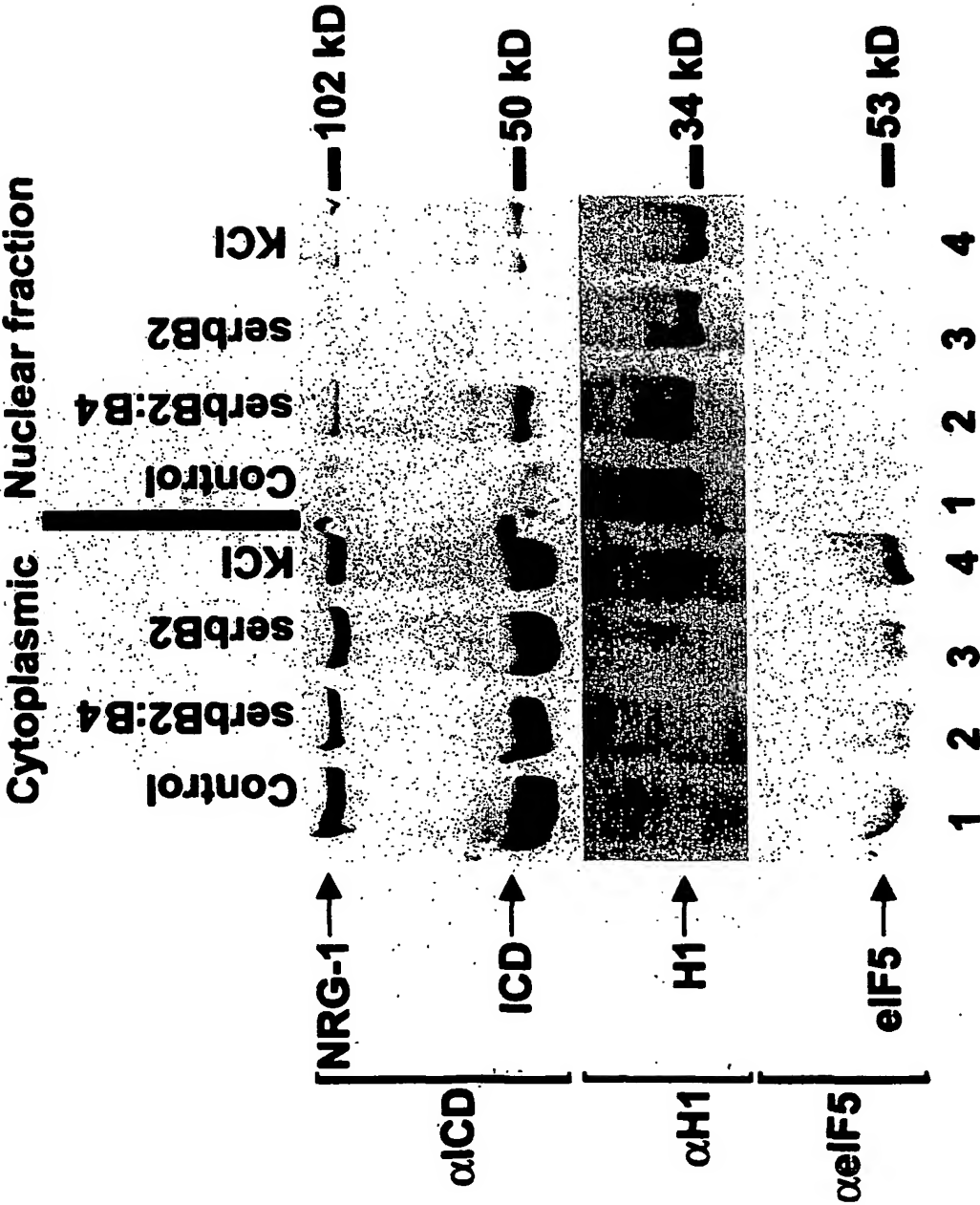


FIGURE 8D





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FIGURE 8E

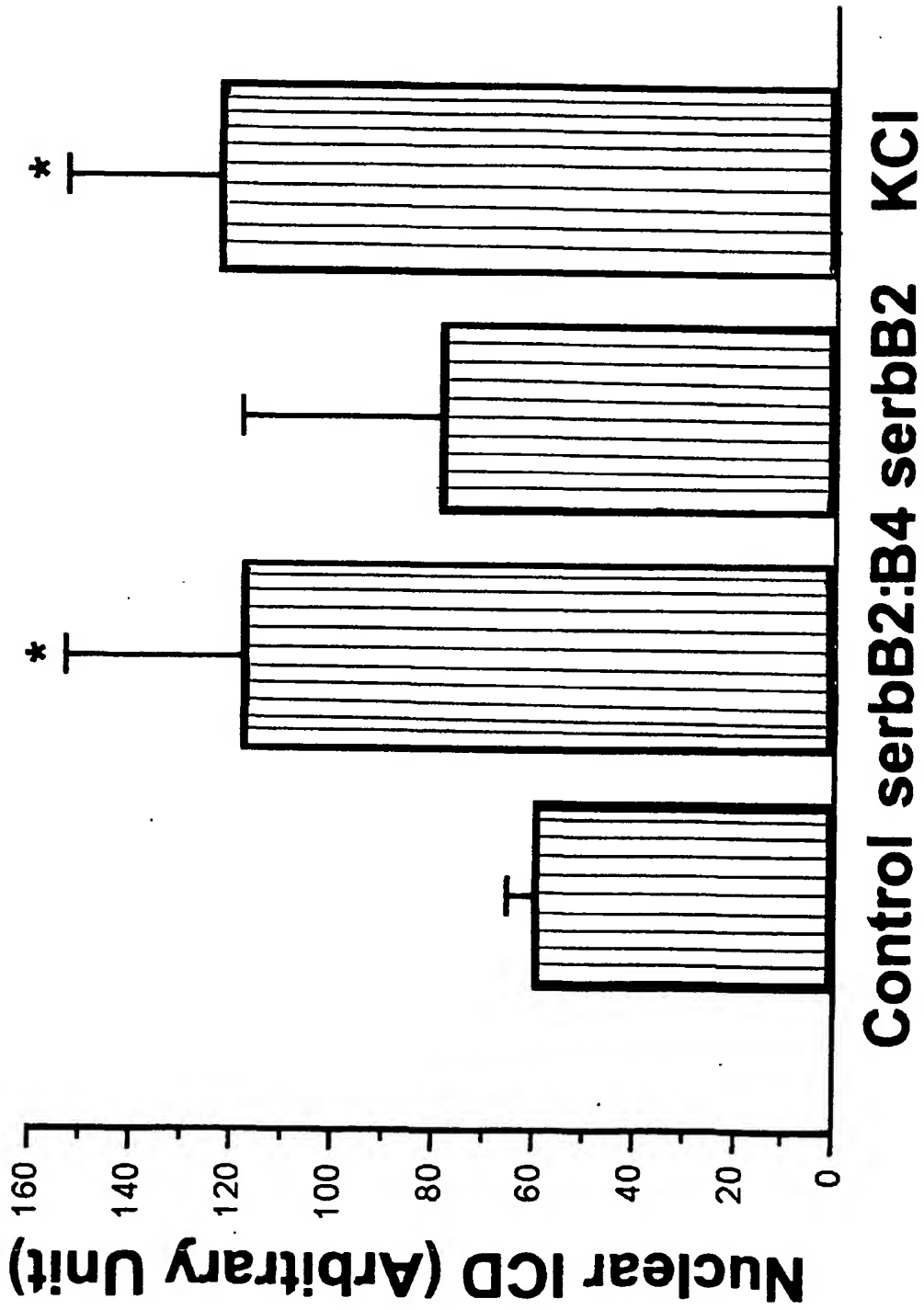
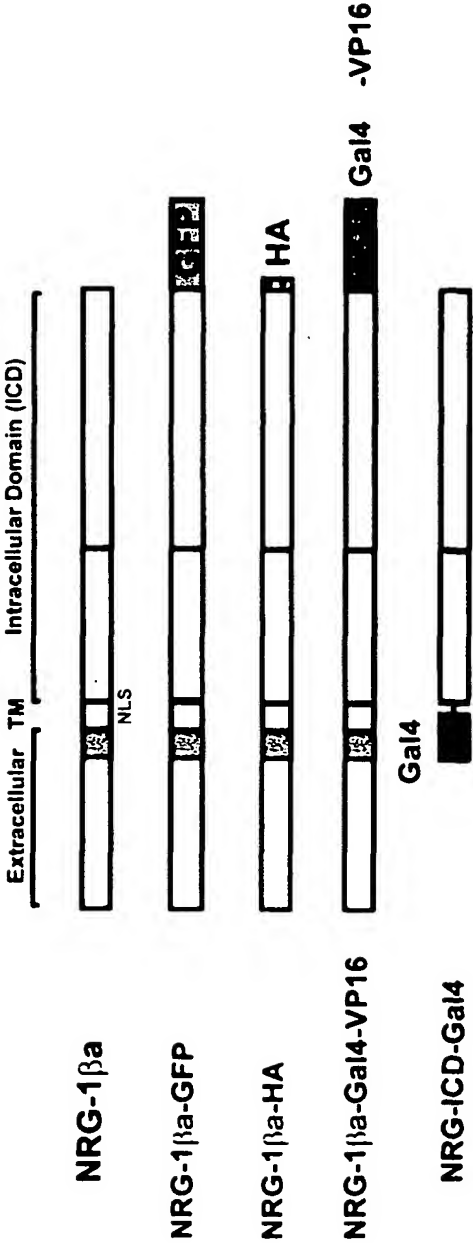


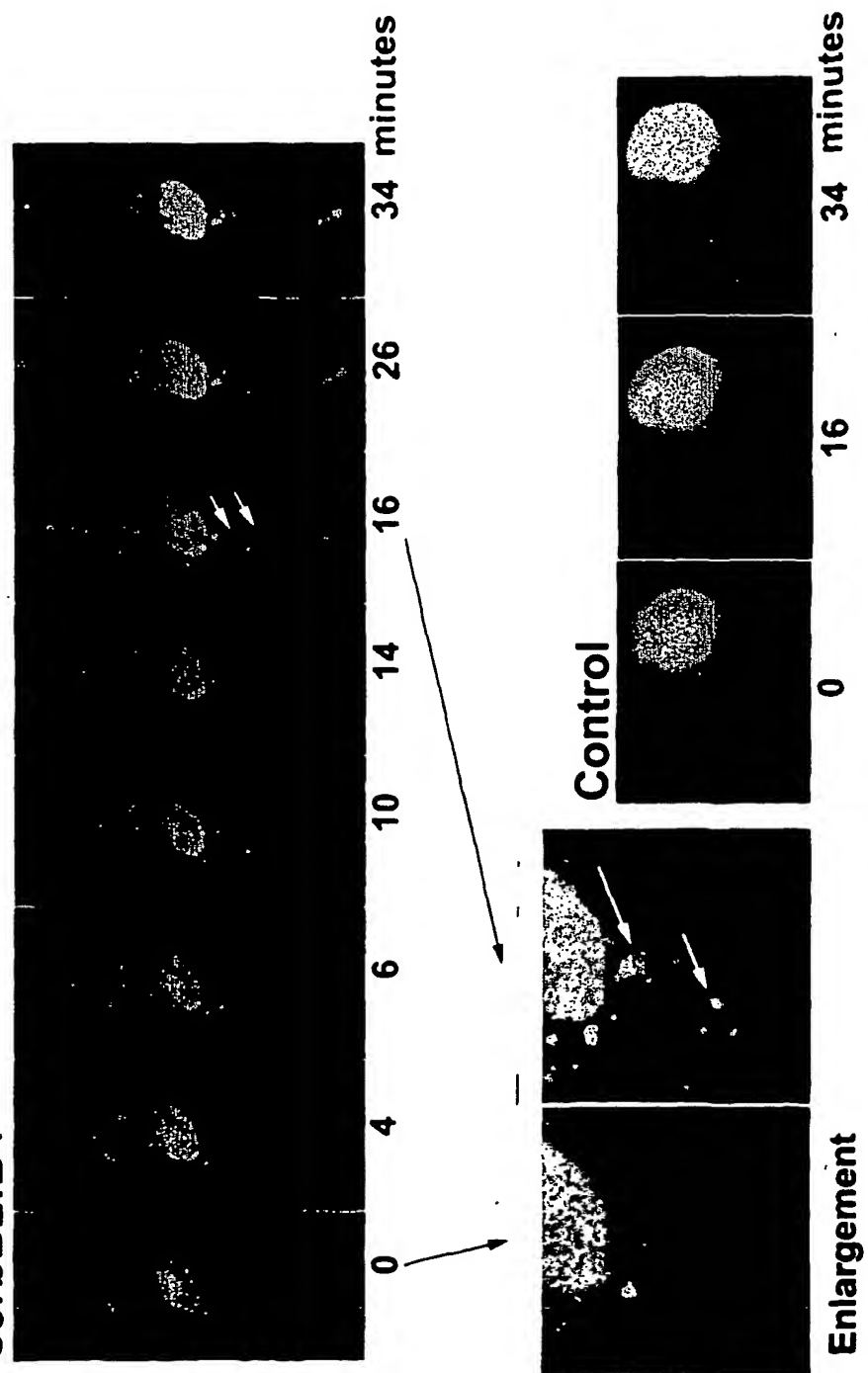
FIGURE 9A



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**FIGURE 9B**

serbB2:B4



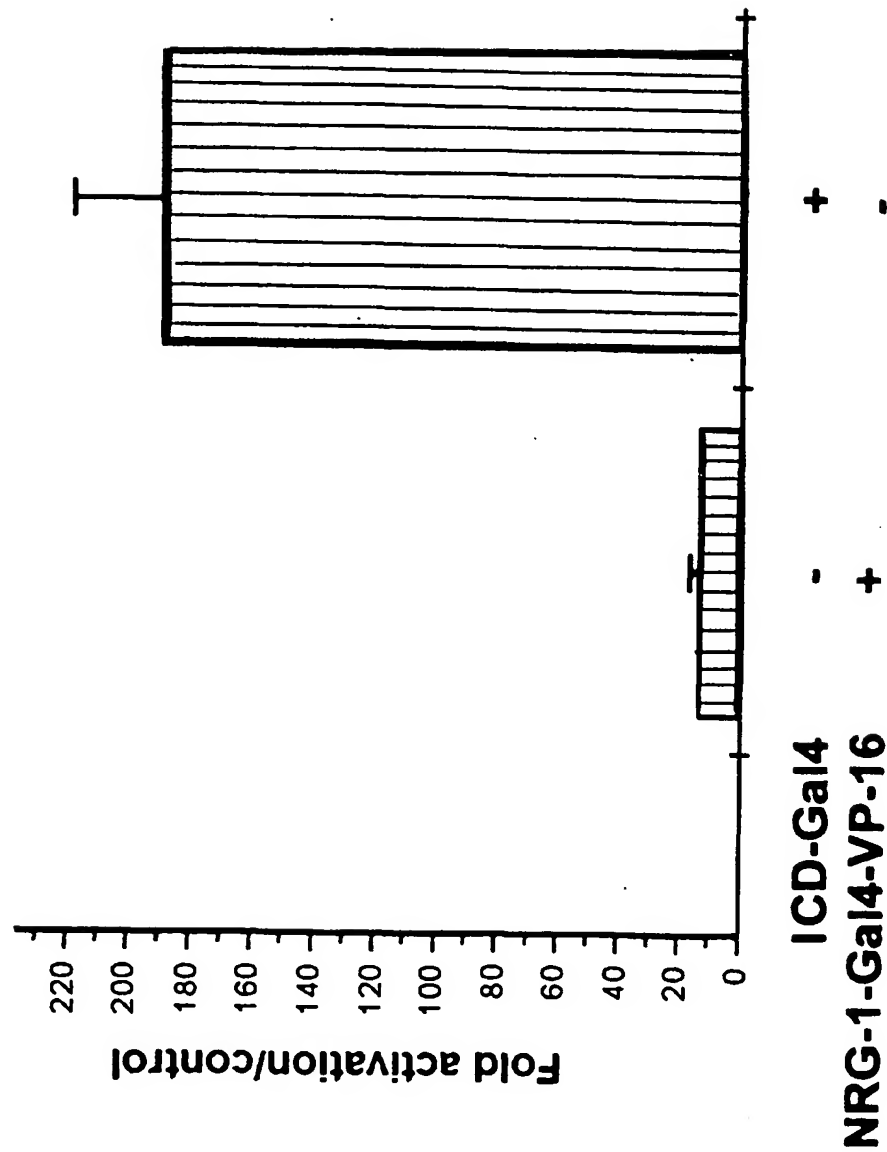
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FIGURE 9C



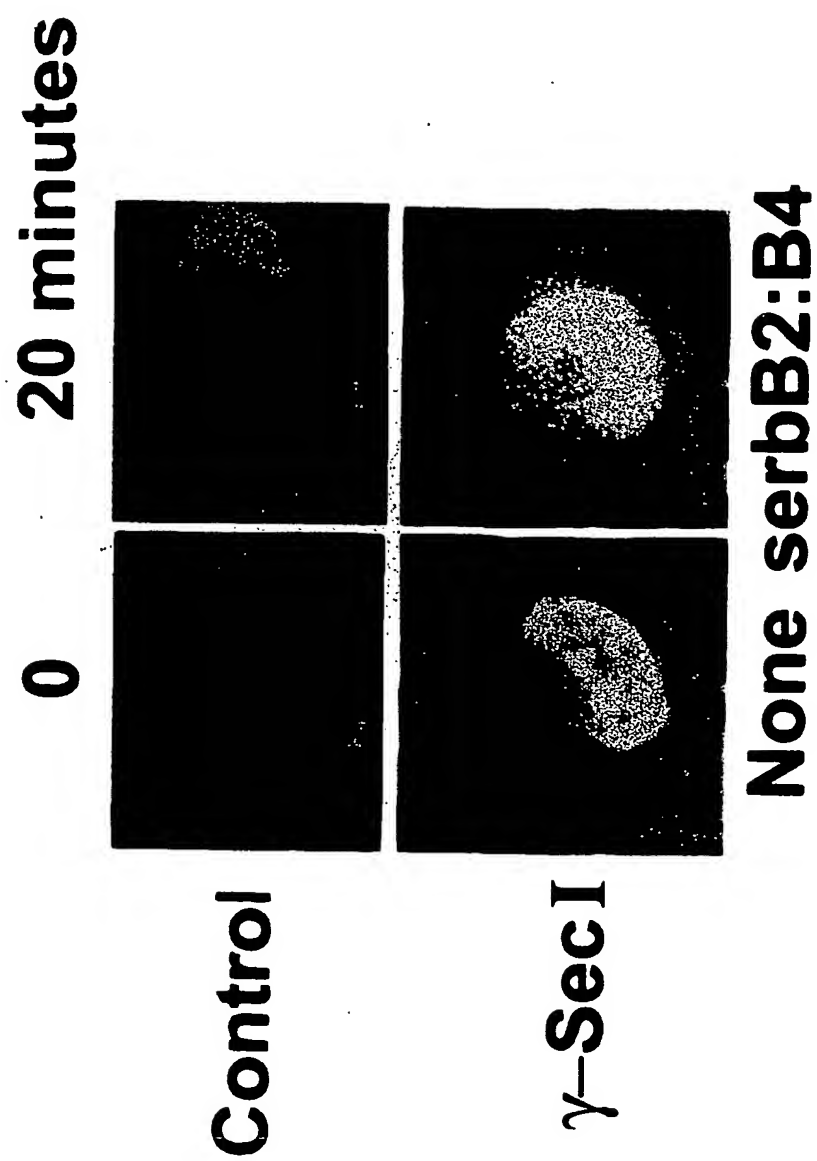
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FIGURE 9D



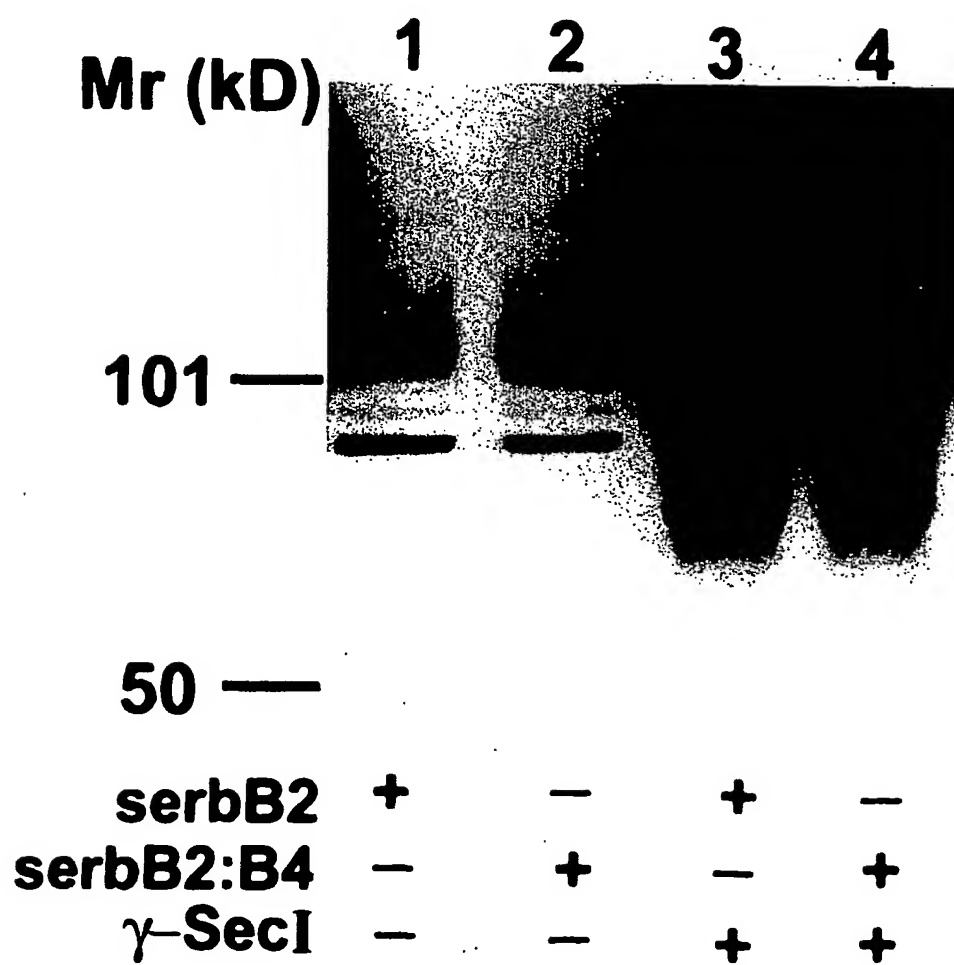
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FIGURE 10A



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FIGURE 10B



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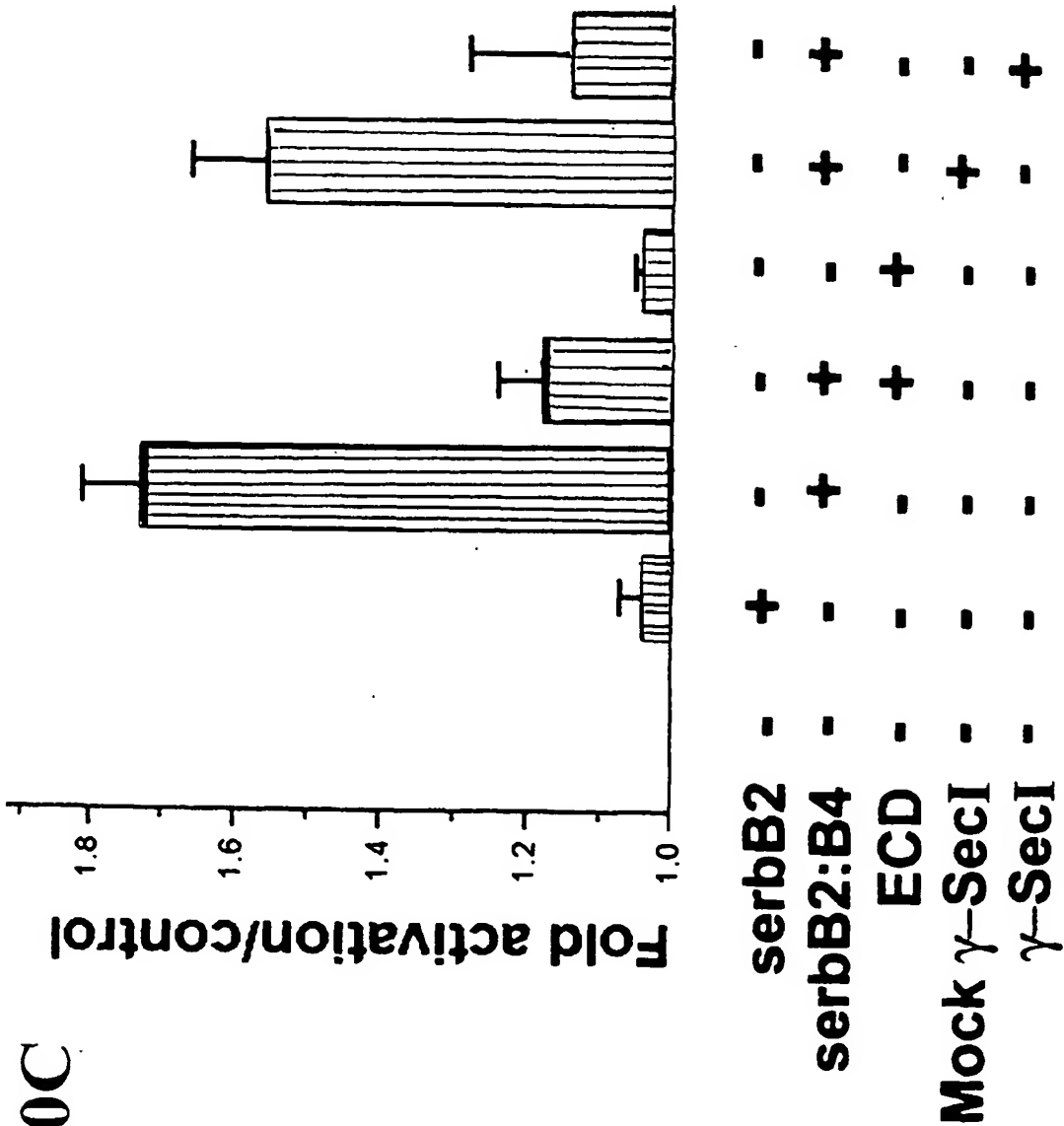
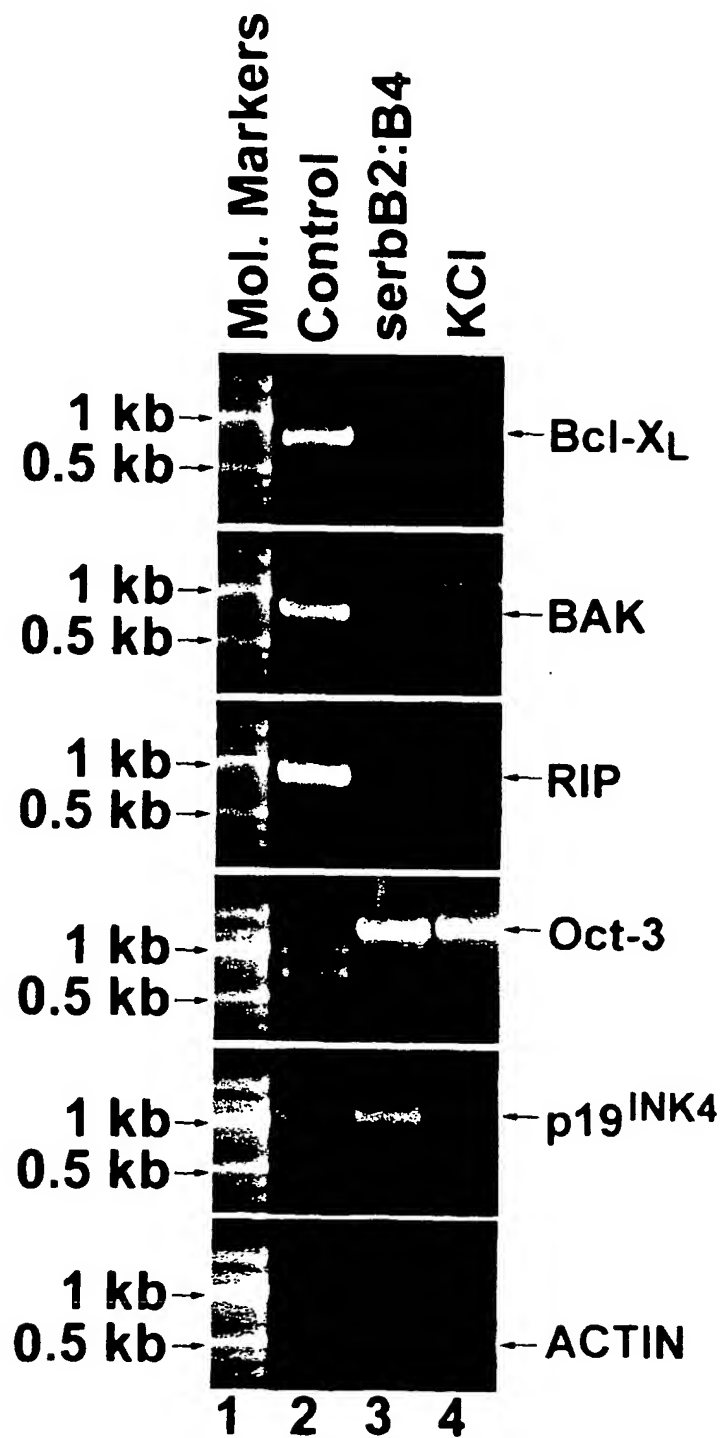


FIGURE 10C



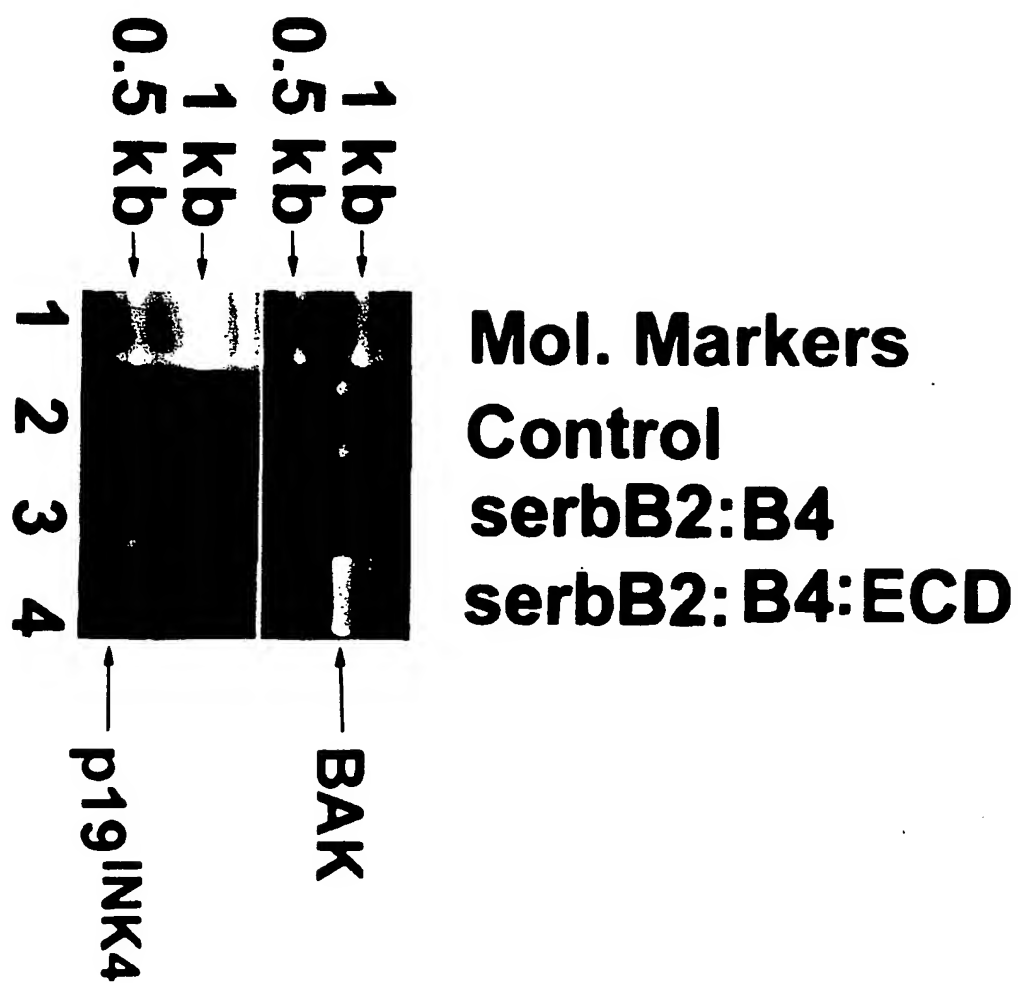
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FIGURE 11A



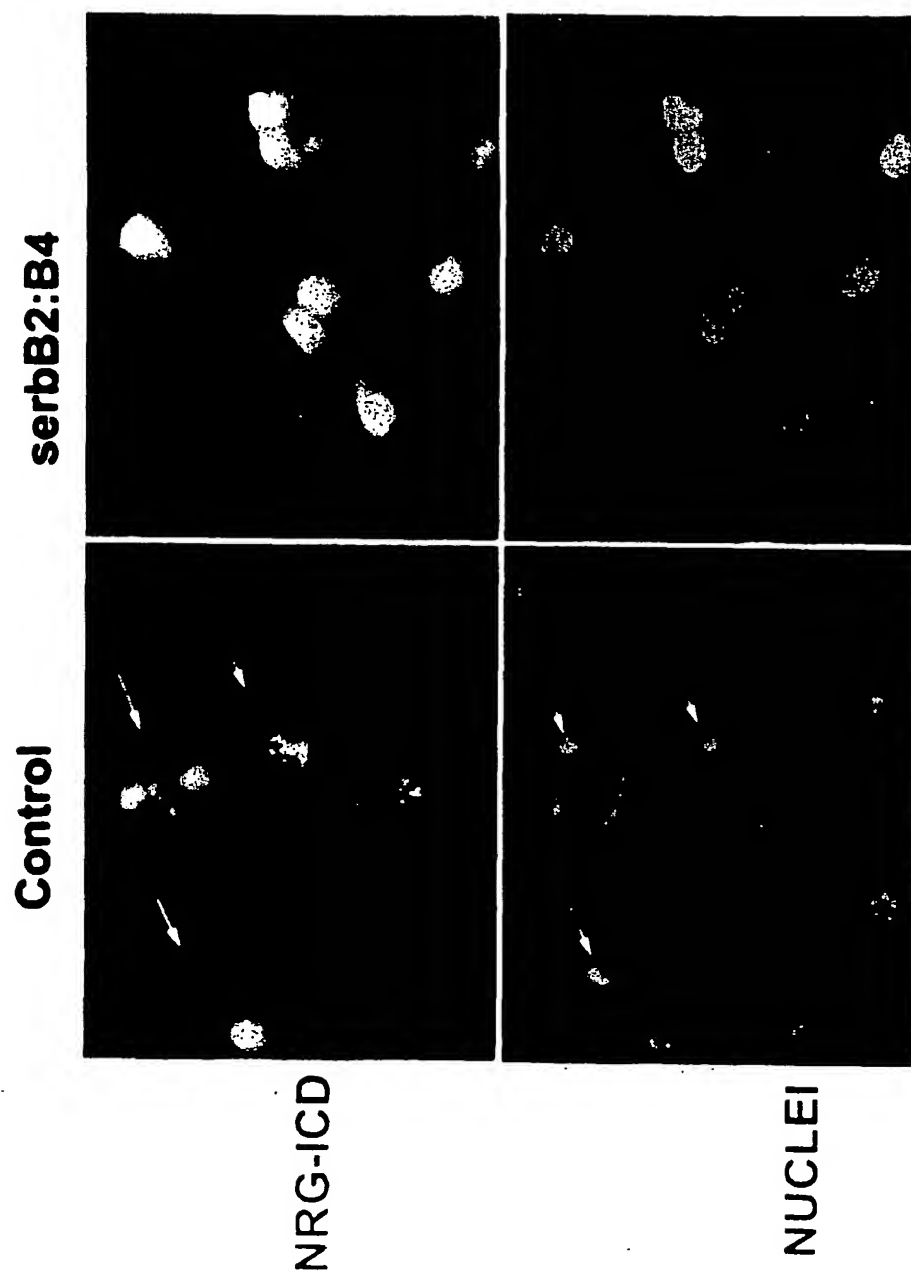
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FIGURE 11B



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FIGURE 11C



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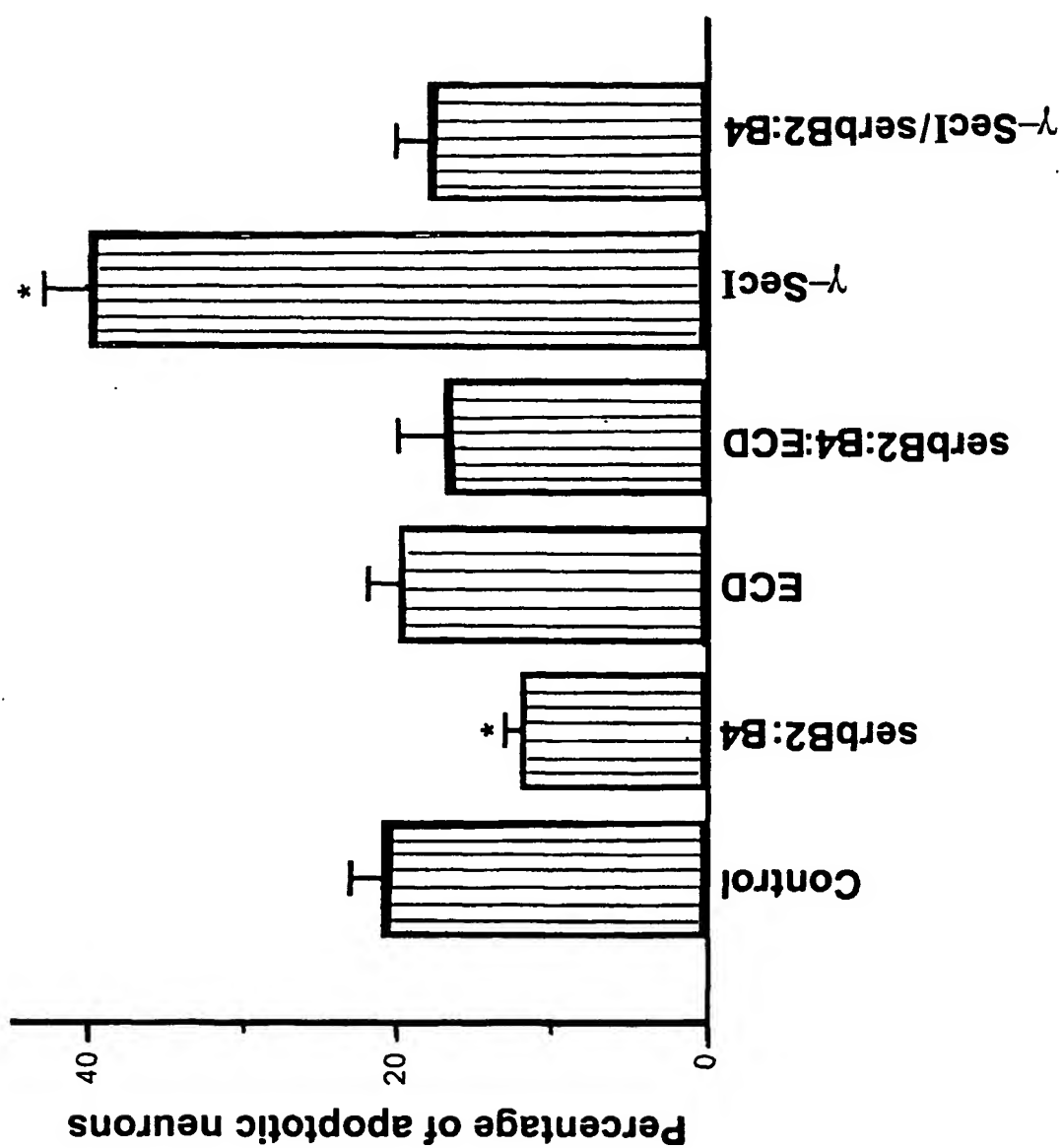


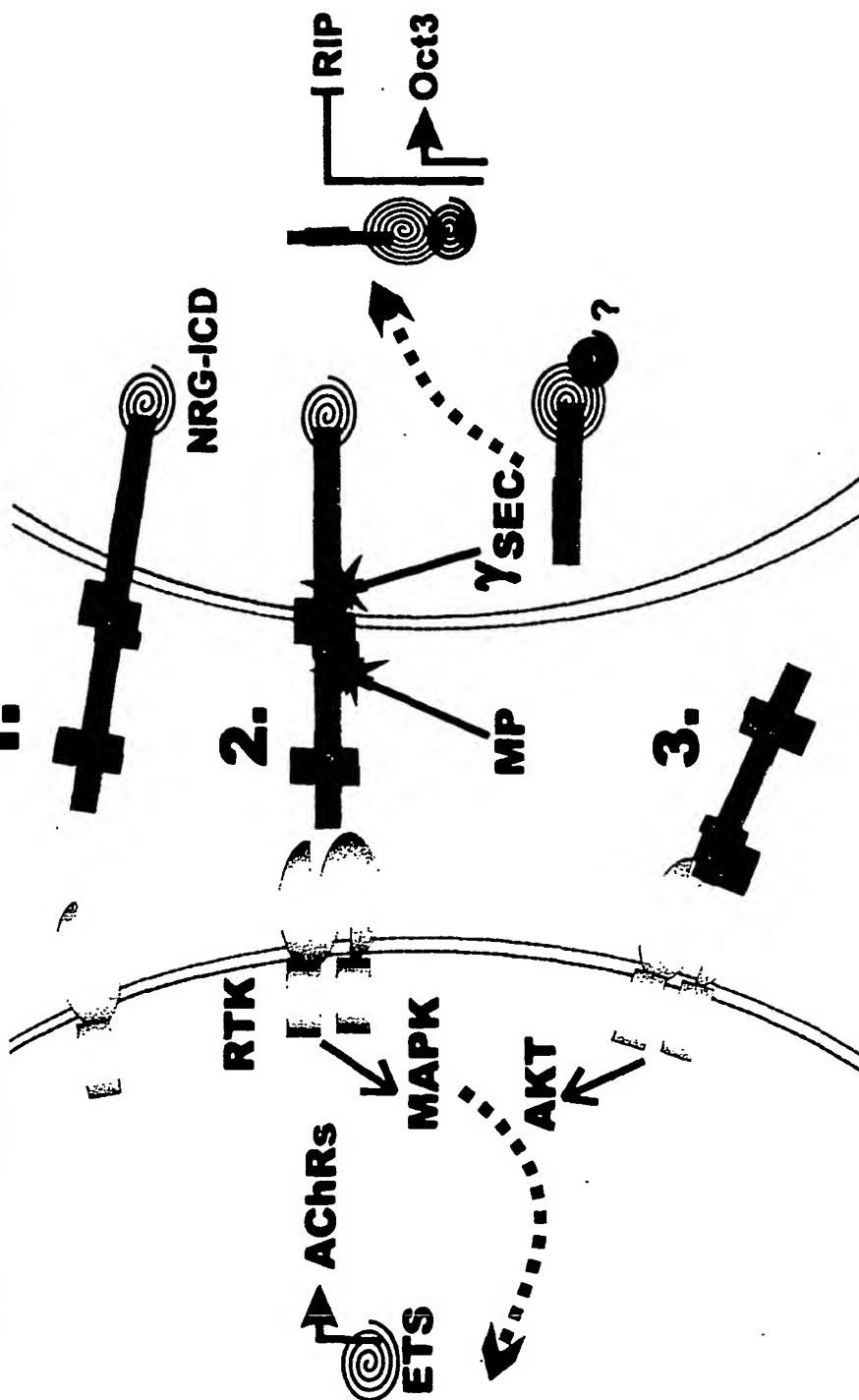
FIGURE 11D

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**FIGURE 12**

**erbB2 and erbB4  
POSITIVE CELL**

**NEUGEGULIN  
POSITIVE CELL**



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